**REMARKS**

Claims 172 to 211 were pending in the subject application. Applicants herein have amended claims 172, 176 to 188, 199, 200, and 211, and have canceled claims 173 to 175, 189, 198, 201, and 210 without disclaimer or prejudice to applicants' right to pursue the subject matter of the canceled claims in this or another application. After entry of this Amendment, claims 172, 176 to 188, 199, 200, and 211 will be pending and under examination.

*Support for Amendments to the Claims*

The subject application is a continuation of U.S. Serial No. 10/346,853, filed January 17, 2003, which is a continuation of U.S. Serial No. 09/100,812, filed June 19, 1998, now U.S. Patent No. 6,573,099 B2, issued June 3, 2003, which claims priority of Australian Provisional Patent Application No. PP2492, filed March 20, 1998 (the "Priority Application"). The amendments to the claims are fully supported in each disclosure.

a) "double-stranded"

Examples of "double-stranded" synthetic genes are replete in each disclosure. Specifically, a number of genetic constructs are described in each disclosure. *See* page 28, line 14, to page 39, line 22. The genetic constructs that each specification describes are ultimately derived from a double-stranded plasmid, such as pCR2.1. *See, e.g.*, page 27, lines 1 to 8. Applicants attach hereto as **Exhibit A** a map of plasmid pCR2.1, which is a commercially available starting plasmid for a number of the "Examples". Additionally, each specification describes blunt-

ended fragments. *See, e.g.*, page 37, lines 6 to 7.

b) "synthetic gene"

Support for this language may be found, *inter alia*, in each specification at page 7, lines 1 to 3.

c) "structural gene sequence"

Support for this language may be found, *inter alia*, in each specification at page 7, lines 5 to 7.

d) "identical"

Support for this language may be found, *inter alia*, in each specification at page 18, lines 17 to 18.

e) "inverted orientation"

Support for this language may be found, *inter alia*, in each specification at page 18, lines 16 to 20.

f) "20-30 consecutive nucleotides"

Support for this language may be found, *inter alia*, in each specification at page 10, lines 15 to 17.

g) "repeating sequence"

Support for this language may be found, *inter alia*, in each specification at page 18, lines 16 to 20.

h) "connected to"

Support for this language may be found, *inter alia*, in each specification at page 11, lines 28 to 29; and page 14, lines 6 to 9.

Applicants have canceled claims 173 to 175, 189, 198, 201, and 210 without disclaimer or prejudice to applicants' right to pursue the subject matter of the canceled claims in this or another application. Accordingly, claims 172, 176 to 188, 199, 200, and 211 are pending and under examination.

**Third-Party Submission Filed Under 37 C.F.R. § 1.99 and Information Disclosure Statement**

The Examiner noted that a third-party submission has been filed under 37 C.F.R. § 1.99 on February 28, 2008 in connection with the subject application. The Examiner indicated that if applicants want to ensure that the information in a third-party submission is considered by the Examiner, applicants should submit the information in an Information Disclosure Statement in compliance with 37 C.F.R. §§ 1.97 and 1.98.

On page 3 of the July 9, 2008 Office Action, the Examiner noted that the references cited in the International Search Reports dated September 27, 2002, March 16, 2001, and March 19, 1999, and the Partial European Search Reports dated November 2, 2007 and June 3, 2005 have been considered and will be listed on any patent resulting from this application.

*Applicants' Response*

Applicants thank the Examiner for pointing out the foregoing, and for the consideration of the information submitted.

For clarity in the record, applicants point out that the references cited in the February 22, 2008 Third Party Submission have been included as items 1 to 4, and 113 to 116 in the Information Disclosure Statement filed April 15, 2008. Applicants also listed items 1 to 4 on page 1 and items 113 to 116 on page 11 of the Form PTO-1449 (Substitute) provided with the April 15, 2008 Information Disclosure Statement.

Applicants also wish to bring to the Examiner's attention several disclosures the consideration of which has not been clearly indicated in the record. Specifically:

- There appear to be uninitialed citations on page 11 of the Form PTO-1449 (Substitute) submitted with applicants' April 15, 2008 Information Disclosure Statement as returned with the July 9, 2008 Office Action. For the Examiner's convenience, applicants have attached hereto as **Exhibit B** a copy of page 11 of the Form PTO-1449 (Substitute) received with the July 9, 2008 Office Action.

- Applicants have not received the Form PTO-1449 (Substitute) submitted with the June 25, 2008 Information Disclosure Statement. For the Examiner's convenience, applicants have attached hereto as **Exhibit C** a copy of the Form PTO-1449 (Substitute) submitted with the June 25, 2008 Information Disclosure Statement.

- Applicants listed a June 3, 2005 European Search Report as item 2 the Form PTO-1449 (Substitute) provided with the June 30, 2005 Information Disclosure Statement. The Examiner struck the European Search Report from the Form PTO-1449 (Substitute) returned with the April 25, 2007 Office Action, without providing any reason for doing so, and without indicating that the June 3, 2005 Search Report was considered.

Applicants respectfully request that the Examiner indicate consideration of the each of the foregoing in any subsequent communication issued by the United States Patent and Trademark Office.

**Election/Restriction**

The Examiner withdrew viral coat protein and viral DNA polymerase in claims 172, 188, and 200 from further consideration pursuant to 37 C.F.R. § 1.142(b), as drawn to a non-elected species, there being no allowable generic or linking claim. The Examiner indicated that applicants timely traversed the restriction (election) requirement in the reply filed on December 29, 2006.

*Applicants' Response*

Applicants thank the Examiner for acknowledging that applicants timely traversed the election requirement in the December 29, 2006 Amendment. Applicants understand that the elected species of "viral RNA polymerase" is being examined, and look forward to the examination of the other species upon allowability of the elected species, pursuant to M.P.E.P. § 809 (8th Ed., 6th Rev., Sept. 2007), *et seq*.

**Claim Rejections Under 35 U.S.C. § 103(a) - Fire et al. Patent taken with Cowsert et al.**

The Examiner rejected claims 172 to 211 under 35 U.S.C. § 103(a) as allegedly unpatentable over U.S. Patent No. 6,506,559 B1 ("Fire et al. Patent") taken with U.S. Patent No. 5,580,767 ("Cowsert et al."). The Examiner's specific rationale is set forth on page 3, line 18, and page 9, line 9, of the July 9, 2008 Office Action.

*Applicants' Response*

In response, without conceding the accuracy of the Examiner's position and in order to expedite prosecution, applicants have amended claims 172, 176 to 188, 199, 200, and 211, and have canceled claims 173 to 175, 189, 198, 201, and 210 without disclaimer or prejudice to applicants' right to pursue the subject matter of the canceled claims in this or another application. Applicants respectfully submit that the amended claims are patentable for at least the reasons which follow.

1. **The invention recited in the claims of the subject application is patentably distinct from the subject matter claimed and disclosed by Fire et al.**

On page 5, lines 7 to 8, of the July 9, 2008 Office Action the Examiner asserted that Fire et al. Patent claims the rejected invention. The Examiner also asserted that an affidavit or declaration is inappropriate under 37 C.F.R. § 1.131(a) when the reference is claiming the same patentable invention, referencing M.P.E.P. § 2306, on page 5, lines 8 to 10, of the July 9, 2008 Office Action.

Applicants maintain that the invention claimed in the subject application is patentably distinct from 1) the subject matter claimed in the issued Fire et al. Patent, and 2) the subject matter disclosed by Fire et al. Specifically, Applicants' claims recite, *inter alia*, a repeating sequence of only 20-30 consecutive nucleotides in length, in the context of mammalian cells. Such a feature is neither claimed nor disclosed by Fire et al. Most importantly, as explained in detail below in sections 4-6 of these Remarks, Applicants' claimed invention incorporating this feature constitutes an unpredictable improvement over the subject matter claimed, as well as the subject matter disclosed by Fire et al.

It is well settled that there is no interference-in-fact when the claimed invention of one party if patentably distinct from the invention of another party. *See, e.g.*, M.P.E.P. § 2301.03. Applicants' claimed invention is clearly patentably distinct from claims 1 and 12 of the issued Fire et al. patent, which is limited, *inter alia*, to "separate" strands. Applicants' claimed invention is also patentably distinct from the entirety of the Fire et al. disclosure, indicating that interfering claims cannot validly be presented in any continuing application of the Fire et al. patent.

In summary, Applicants respectfully submit that the invention claimed in the subject application is patentably distinct over Fire et al. Therefore, the Fire et al. Patent as well as any Fire et al. continuing application cannot be a proper reference against the subject application, and the subject application should be advanced to allowance without delay for reasons which follow.

**2. Fire et al. Patent is not prior art to the claimed invention.**

As an initial matter, Fire et al. Patent is not prior art to the subject application, as applicants have previously explained. To summarize, the amended claims are entitled to the priority of the March 20, 1998 filing date of Australian Provisional Patent Application No. PP2492. Fire et al. Patent issued from an application submitted to the United States Patent and Trademark Office on December 23, 1998, *i.e.* after the priority date of the subject application.

Fire et al. Patent claims the benefit of U.S. Provisional Application No. 60/068,562, filed December 23, 1997 ("Fire et al. Provisional"). However, Fire et al. Provisional discloses less than Fire et al. Patent. Applicants attach hereto as **Exhibit D** a copy of Fire et al. Patent marked-up to show differences from Fire et al. Provisional.

The July 9, 2008 Office Action cites numerous portions of Fire et al. Patent which do not appear in Fire et al. Provisional. The rejections which rely on disclosures not in Fire et al. Provisional are clearly improper. However, in the interests of compact prosecution, applicants will refer to the Fire et al. *Provisional* in connection with the rejection under 35 U.S.C. § 103(a) over Fire et al. *Patent* in view of Cowsert et al.

**3. The combination of Fire et al. Provisional and Cowsert et al. does not disclose synthetic genes with a "stuffer fragment" as recited in the amended claims.**

The pending claims recite a "stuffer fragment which consists of nucleotides and which is in between and links the first and

second structural gene sequences". Activation of the double-stranded promoters of the claimed invention causes transcription of single-stranded oligonucleotides comprising a region corresponding to the "first structural gene sequence", a region corresponding to the "stuffer fragment" and a region corresponding to the "second structural gene", wherein only 20-30 nucleotides of the region corresponding to the "first structural gene sequence" and only 20-30 nucleotides of the region corresponding to the "second structural gene" are complementary. The region corresponding to the "stuffer fragment" is a distinct region.

Applicants note that the July 9, 2008 Office Action asserted that an arbitrary number of nucleotides "can be arbitrarily considered to be a stuffer fragment" at page 6, lines 4 to 6. However, being separate recited elements of the same claim compels the conclusion that "stuffer fragment" has a separate meaning from the meaning of "structural gene sequence". *See, e.g., Tandon Corp. v. U.S. Int'l Trade Comm'n.*, 831 F.2d 1017, 1023 (Fed. Cir. 1987) ("There is presumed to be a difference in meaning and scope when different words or phrases are used in separate claims.")(emphasis added); *see also Gen. Am. Transp. Corp. v. Cryo-Trans, Inc.*, 93 F.3d 766, 770 (Fed. Cir. 1996)(claim terms shall not be construed so as to be "superfluous")[1]. Therefore, the "first structural gene sequence", the "second structural gene sequence", and "stuffer fragment" must be construed to each be a distinct element.

There is no disclosure that the "transgene" or "expression vector" on page 7, lines 13 to 15, or page 11, lines 19 and 20,

[1] Copies of these cases were included with the July 11, 2008 Amendment submitted in connection with Merged Reexamination Nos. 90/007,247 and

of Fire et al. Provisional has a distinct "stuffer fragment". Even if the Fire et al. Provisional can be interpreted to suggest that the "transgene" or "expression vector" can be used to make the "single self-complementary RNA strand," it does not disclose whether any distinct portion corresponding to a "stuffer fragment" is present in the "transgene" or "expression vector." Indeed, an interpretation that the "transgene" or "expression vector" of the Fire et al. Provisional necessarily contains a central "stuffer fragment" is arbitrary and hints of hindsight bias.

Cowsert et al. does not disclose a construct with a "stuffer fragment." Therefore, the combination of Cowsert et al. with Fire et al. Provisional fails to remedy the aforementioned deficiency of the Fire et al. Provisional. Consequently, the amended claims are patentable over the combination of cited references.

**4. The combination of Fire et al. Provisional and Cowsert et al. does not teach or suggest synthetic genes with "a repeating sequence of only 20-30 consecutive nucleotides in length" identical to the target.**

The combination of Fire et al. Provisional and Cowsert et al. cannot render obvious the amended claims because the combination does not teach or suggest synthetic genes with "a repeating sequence of only 20-30 consecutive nucleotides in length", as claimed.

While the July 9, 2008 Office Action disregarded this element after broadly construing applicants' previously pending claims, the amended claims herein unambiguously require the specified

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90/008,096, filed October 4, 2004 and May 18, 2006, respectively.

range of identity. The combination of cited references fails to teach the specified range, *i.e.* the combination of cited references fails to teach all of the elements of the pending claims. Consequently, the amended claims are patentable over the combination of cited references.

**5. The specification calls out that the range of "20-30 consecutive nucleotides" is preferred.**

Recognizing applicants' intent to claim the range of identity, the July 9, 2008 Office Action asserted that:

> ". . . there is nothing in the specification that would lead one of ordinary skill in the art to the teaching in the post-filing reference. The specification did not disclose the observation disclosed in the post-filing reference." (Page 9, lines 6 to 9, of the July 9, 2008 Office Action)

The subject application as originally filed and the Priority Application do in fact *call out* the recited range as preferred in the context of mammalian cells. Specifically, the specification provides that:

> "Preferred structural gene components of the synthetic gene of the invention comprise at least about 20-30 nucleotides in length derived from a viral DNA polymerase, viral RNA polymerase, viral coat protein or visually-detectable gene, more particularly an RNA polymerase gene derived from a virus selected from the list comprising BEV[2], Sindbis alphavirus, HIV-1, bovine herpes virus and HSV1[3] or a visually-detectable gene which is involved in determining pigmentation, cell death or other external phenotype on a cell, tissue, organ or organism, amongst others." (Page 10, lines

[2] Each specification defines "BEV" as "*bovine* enterovirus" (page 7, lines 26 to 27; emphasis added).
[3] Each specification defines "HSVI" as "herpes simplex virus I" (page 7, line 29).

15 to 21; emphasis added)

Clearly, the specification *calls out* as "preferred" the range of 20-30 nucleotides as recited in the amended claims (single underlined language above). Moreover, the range is called out "more particularly" in the context of viruses which all infect *mammalian cells* (doubly underlined language above).

Thus, the specification *calls out* as a "preferred" embodiment the range required by the pending claims.

Notwithstanding and in addition to the fact that applicants' application as filed disclosed the claimed range to be preferred, advantages of the claimed range must be considered even if the application had not done so. Contrary to the July 9, 2008 Office Action, M.P.E.P. § 716.02(f) (8th Ed., 6th Rev., Sept. 2007) unambiguously commands that, "evidence and arguments directed to advantages not disclosed in the specification cannot be disregarded", citing *In re Chu*, 66 F.3d 292, 298-99, 36 U.S.P.Q.2d 1089, 1094-95 (Fed. Cir. 1995). The same section of the M.P.E.P further explains that "[t]he specification need not disclose proportions or values as critical for applicants to present evidence showing the proportions or values to be critical" (emphasis added), citing *In re Saunders*, 444 F.2d 599, 607, 170 U.S.P.Q. 213, 220 (C.C.P.A. 1971). Applicants have attached as **Exhibit E** a copy of *In re Saunders* for the convenience of the Examiner. The Court of Customs and Patent Appeals in *Saunders* explained that:

> "[a]ppellants' evidence may not be disregarded simply because of the manner in which the now-claimed processes were denominated in the original application. To rule otherwise would let form triumph

> over substance, substantially eliminating the right of an applicant to retreat to an otherwise patentable species merely because he erroneously thought he was first with the genus when he filed."

*Id.*; emphasis added.

In summary, applicants maintain that the specification calls out the range of "20-30 consecutive nucleotides" in the context of mammalian cells, and the patent law requires consideration of the unpredictable advantages of the range.

**6. Paul et al. evidences that "20-30 consecutive nucleotides" is the unpredictably advantageous range of identity in the context of mammalian cells.**

Paul et al. indicate that:

> "The use of this 'RNA interference' (RNAi) in mammalian studies had lagged well behind its utility in lower animals because uninterrupted RNA duplexes longer than 30 base pairs trigger generalized cellular responses through activation of dsRNA-dependent protein kinases. Recently it was demonstrated that RNAi can be made to work in cultured human cells by introducing shorter, synthetic duplex RNAs (~20 base pairs) through liposome transfection." (Abstract, lines 6 to 11; internal references omitted; emphasis added)

Paul et al. described as the lower limit of the range of identity to a target in mammalian cells to be 20 consecutive nucleotides (single underline) and the upper limit of the range of identity to a target in mammalian cells to be 30 consecutive nucleotides (double underline). The advantage of this range in mammalian cells as recited in the amended claims could not have been predicted from any of the cited prior art.

Accordingly, applicants maintain that the rejection of the previously presented claims under 35 U.S.C. § 103(a) as allegedly obvious over Fire et al. taken with Cowsert et al. cannot be applied to the amended claims.

**Claim Rejections Under 35 U.S.C. § 103(a) - Agrawal et al. in view of Kool and Cowsert et al.**

The Examiner rejected claims 172 to 211 under 35 U.S.C. § 103(a) as allegedly unpatentable over PCT International Publication No. WO 94/01550 ("Agrawal et al.") in view of U.S. Patent No. 5,514,546 ("Kool") and U.S. Patent No. 5,580,767 ("Cowsert et al."). The Examiner's specific rationale is set forth on page 9, line 11, to page 11, line 22, of the July 9, 2008 Office Action.

*Applicants' Response*

In response, without conceding the accuracy of the Examiner's position and without prejudice to applicants' right to pursue the subject matter of the claims in this or another application, applicants have amended claims 172, 176 to 188, 199, 200, and 211, and have canceled claims 173 to 175, 189, 198, 201, and 210 without disclaimer or prejudice to applicants' right to pursue the subject matter of the canceled claims in this or another application. Furthermore, applicants maintain that the rejection cannot apply to the amended claims for at least the reasons given below.

**1. The combination of Agrawal et al., Kool, and Cowsert et al. do not disclose a "stuffer fragment".**

For purposes of clarity in this discussion, applicants provide the following discussion in order to assist in an understanding of the meaning conveyed to persons skilled in the art of certain terms. To assist in this, applicants attach hereto as **Exhibit F** a diagram showing a double-stranded genetic construct, a "single self-complementary RNA strand," and a self-hybridized RNA. The term "self-complementary" is an adjective and, in relevant context, provides information about the single RNA strand. "Self-complementary" informs the reader that the single RNA strand has nucleotides that have the ability to hybridize to other nucleotides in the strand, under appropriate conditions. It does not inform the reader of, *inter alia*, whether some or all nucleotides have this ability, and whether any different segments are present. The term "self-complementary" also does not indicate whether the single RNA strand is self-hybridized.

A self-complementary single-stranded transcript may form a "hairpin" structure when it is self-hybridized. A hairpin oligonucleotide usually has a non-hybridized portion termed a "loop." Importantly, the "loop" results *regardless* of whether the nucleotides constituting the "loop" are self-complementary or not.

In reference to pages 15, lines 12 to 16, and Figure 1 of Agrawal et al., the July 9, 2008 Office Action asserts that "[t]he loop is considered to be a 'stuffer' sequence" (page 9, line 22, of the July 9, 2008 Office Action). The "stuffer fragment" element of the pending claims *cannot* be construed to read on a "loop." because the "loop" of the nucleotides of Agrawal et al. is a

*single-stranded segment*.

In summary, neither Agrawal et al., Kool, and Cowsert et al. nor the combination thereof teach or suggest a "stuffer fragment", and therefore cannot render obvious applicants' invention.

**2. The combined disclosure of Agrawal et al., Kool, and Cowsert et al. does not teach synthetic genes with "a repeating sequence of only 20-30 consecutive nucleotides" identical to the target.**

The combination of Agrawal et al., Kool, and Cowsert et al. also fails to render obvious the amended claims because the combination does not teach or suggest synthetic genes with "a repeating sequence of only 20-30 consecutive nucleotides", as claimed.

**3. Paul et al. evidences that "20-30 consecutive nucleotides" is the unpredictably advantageous range of identity in the context of mammalian cells.**

As discussed above, advantages of the range in mammalian cells of 20-30 contiguous nucleotides as recited in the amended claims could not have been predicted from the prior art.

Accordingly, applicants maintain that the rejection of the previously presented claims under 35 U.S.C. § 103(a) as allegedly obvious over Agrawal et al. taken with Kool and Cowsert et al. cannot be applied to the amended claims.

**Double Patenting Rejection**

a) The Examiner rejected claims 172 to 211 on the ground of nonstatutory obviousness-type double patenting as allegedly

unpatentable over claims 2, 4 to 6, 11, 13 to 15, and 19 to 22 of U.S. Patent No. 6,573,099 B1.

b) The Examiner provisionally rejected claims 172 to 211 on the round of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 48, 107, 110, 111, 114 to 136, 138, and 146 to 149 of copending Application No. 10/646,070.

c) The Examiner provisionally rejected claims 172 to 211 on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 56, 60, 62, 65 to 101, and 107 of copending Application No. 09/646,807.

d) The Examiner provisionally rejected claims 172 to 211 on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 34 and 88 to 133 of copending Application No. 10/821,726.

e) The Examiner provisionally rejected claims 172 to 211 on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claims 44, 77 to 100, 102, 104 to 113, and 142 to 144 of copending Application No. 10/821,710.

f) The Examiner asserted that claims 172 to 211 are directed to an invention not patentably distinct from claims 56, 60, 62, 65 to 101, and 107 of commonly assigned application 09/646,807, specifically, for the reasons set forth in the July 9, 2008 Office Action under the provisional obviousness-type double patenting rejection.

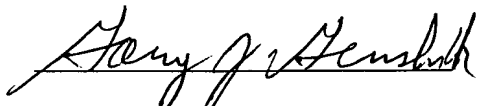
*Applicants' Response*

Applicants thank the Examiner for his careful review and for his notation of applicants' request that the rejections be held in abeyance pending indication of allowable subject matter in the subject application. Applicants respectfully maintain their requests.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Amendment. However, if any other fee is required authorization is hereby given to charge the amount of such additional fee to Deposit Account No. 03-3125.

Respectfully submitted,



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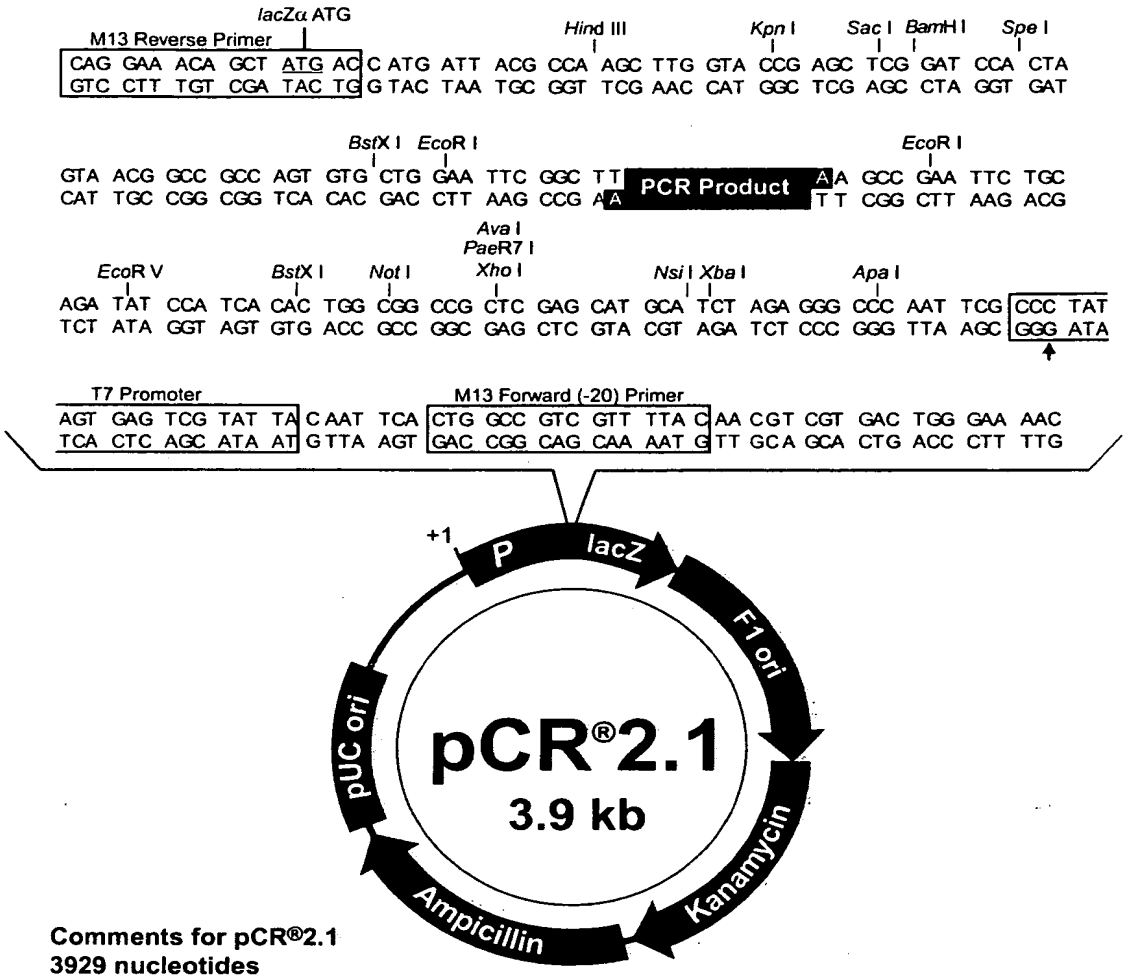
I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to:

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450



Gary J. Gershik Date
Reg. No. 39,992

# EXHIBIT A




**Comments for pCR®2.1**
**3929 nucleotides**

*LacZα* gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

Invitrogen™
life technologies

Applicants: Michael Wayne Graham
and Robert Norman Rice
Serial No.: 10/759,841
Filed: January 15, 2004
**Exhibit A**

# EXHIBIT B

# EXHIBIT D

US006506559B1

(12) **United States Patent**
**Fire et al.**

(10) **Patent No.: US 6,506,559 B1**
(45) **Date of Patent: *Jan. 14, 2003**

(54) **GENETIC INHIBITION BY DOUBLE-STRANDED RNA**

(75) Inventors: **Andrew Fire**, Baltimore, MD (US); **Stephen Kostas**, Chicago, IL (US); **Mary Montgomery**, St. Paul, MN (US); **Lisa Timmons**, Lawrence, KS (US); **SiQun Xu**, Ballwin, MO (US); **Hiroaki Tabara**, Shizuoka (JP); **Samuel E. Driver**, Providence, RI (US); **Craig C. Mello**, Shrewsbury, MA (US)

(73) Assignee: **Carnegie Institute of Washington**, Washington, DC (US)

( * ) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/215,257**

(22) Filed: **Dec. 18, 1998**

**Related U.S. Application Data**

(60) Provisional application No. 60/068,562, filed on Dec. 23, 1997.

(51) **Int. Cl.**[7] ..... **C12Q 1/68**; C12N 15/85
(52) **U.S. Cl.** ..... **435/6**; 435/91.1; 435/325
(58) **Field of Search** ..... 514/44; 435/6, 435/91.1, 325

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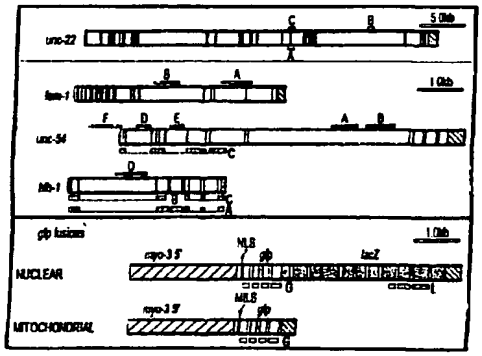
(List continued on next page.)

*Primary Examiner*—Andrew Wang
*Assistant Examiner*—Karen A Lacourciere
(74) *Attorney, Agent, or Firm*—Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

**22 Claims, 5 Drawing Sheets**




Applicants: Michael Wayne Graham and Robert Norman Rice
Serial No.: 10/759,841
Filed: January 15, 2004
**Exhibit D**

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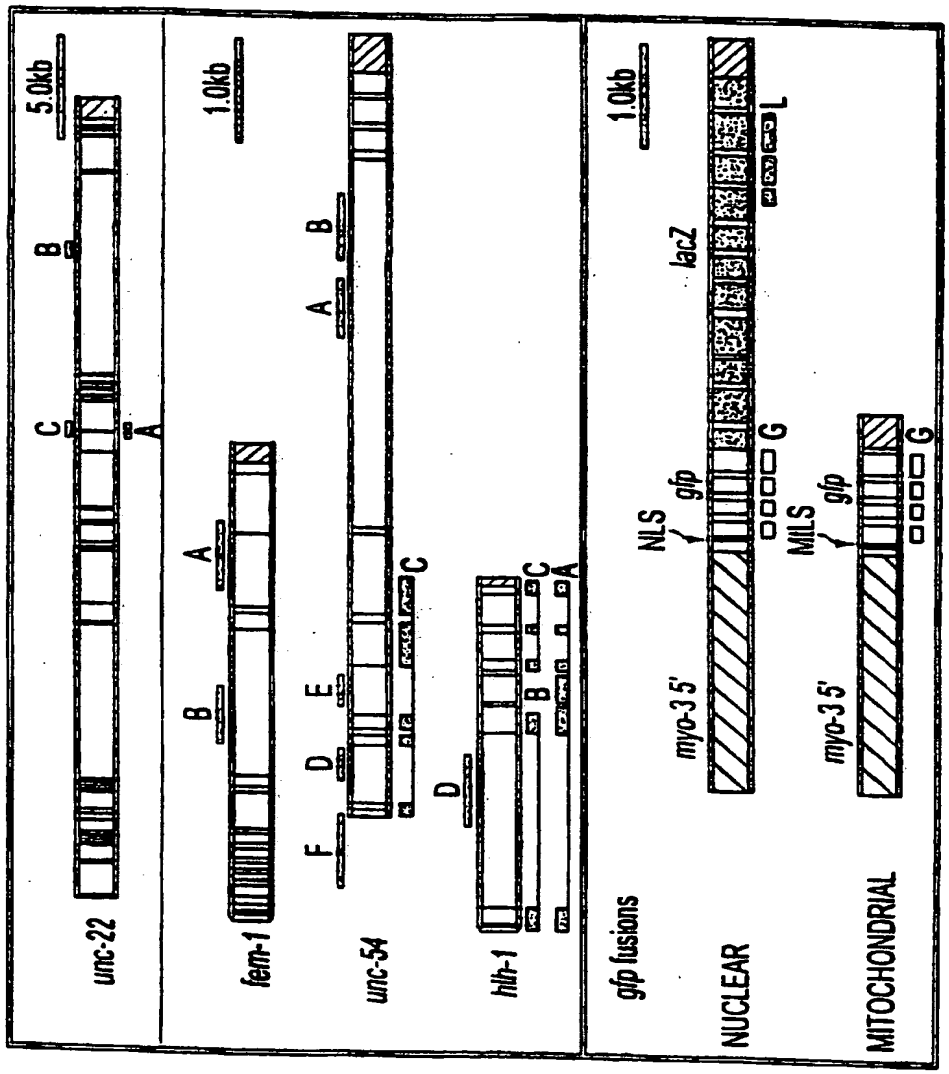
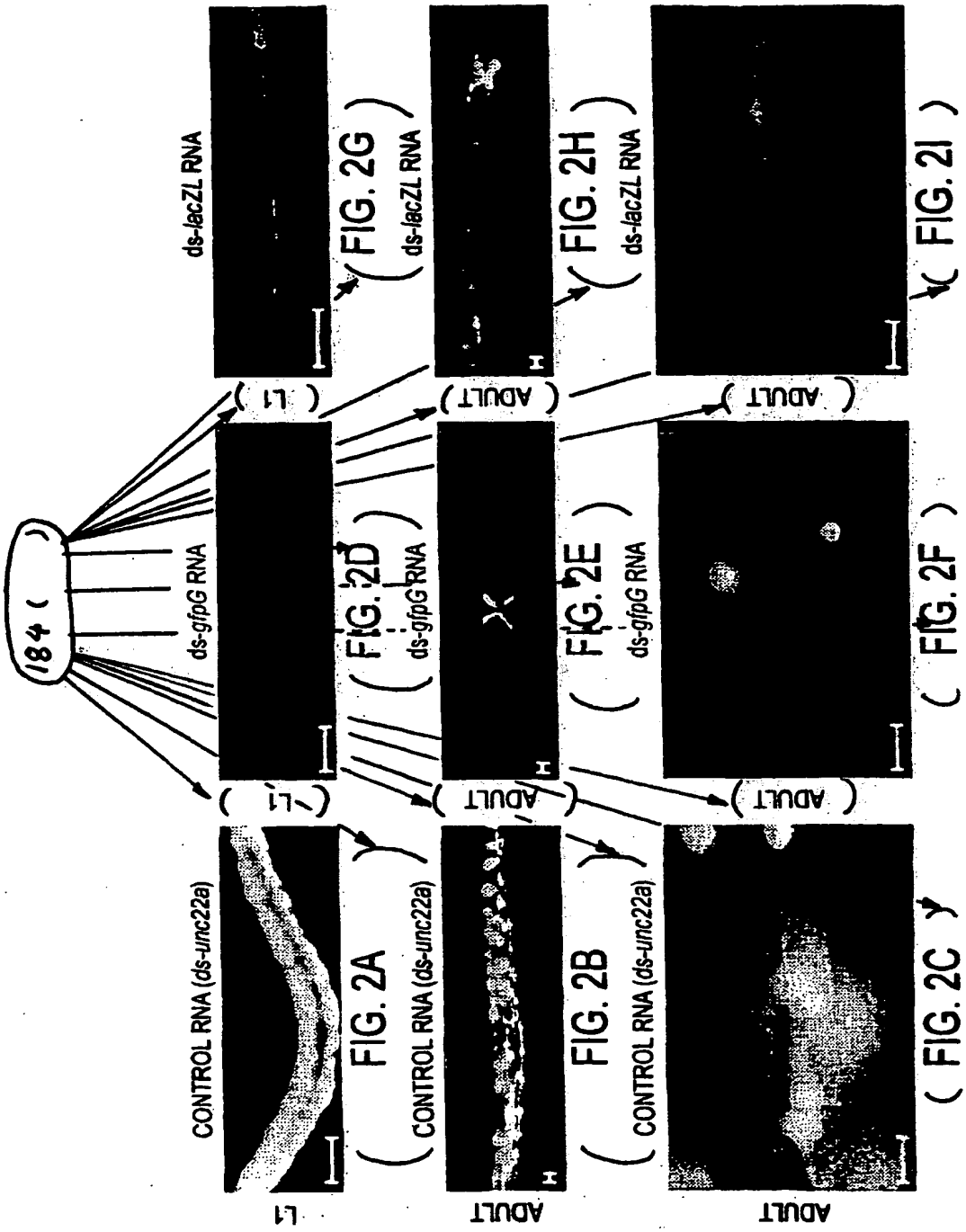

5.0kb
C
B
A
unc-22
1.0kb
B
A
fem-1
F
D
E
A
B
C
unc-54
D
hlh-1
B
A
C
A
gfp fusions
1.0kb
NLS
gfp
myo-3 5'
lacZ
L
G
NUCLEAR
MLS
gfp
myo-3 5'
G
MITOCHONDRIAL

FIG. 1


184
CONTROL RNA (ds-unc22a)
ds-gfpG RNA
ds-lacZL RNA
L1
ADULT
ADULT
FIG. 2A
FIG. 2B
FIG. 2C
FIG. 2D
FIG. 2E
FIG. 2F
FIG. 2G
FIG. 2H
FIG. 2I

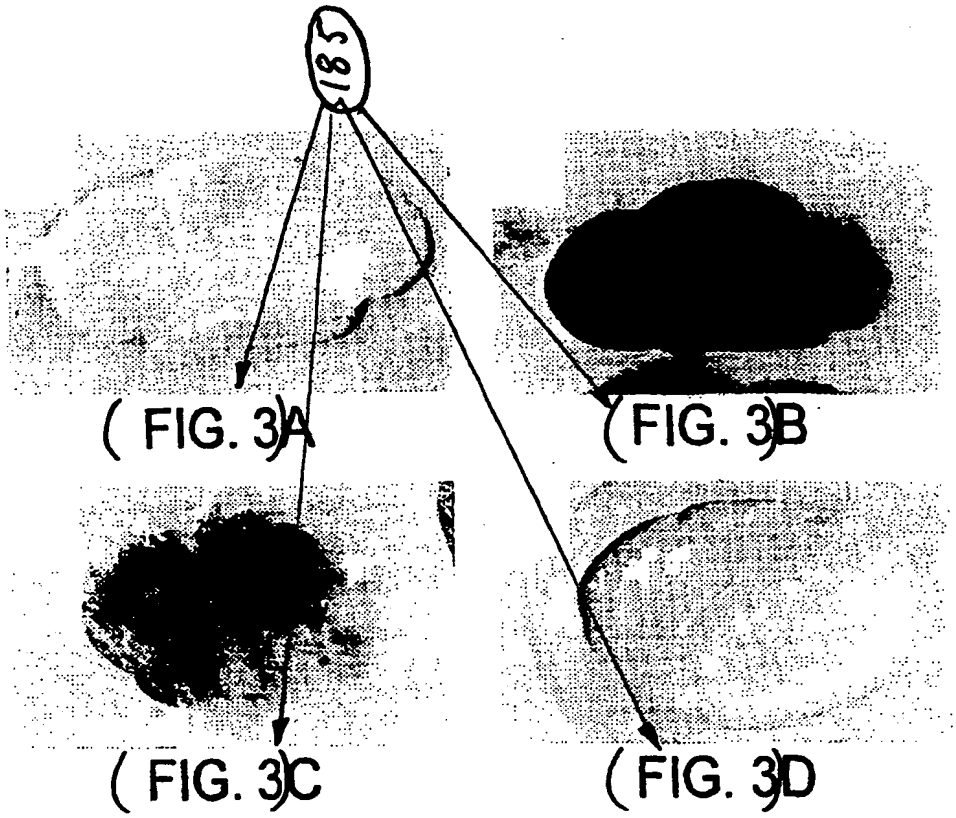
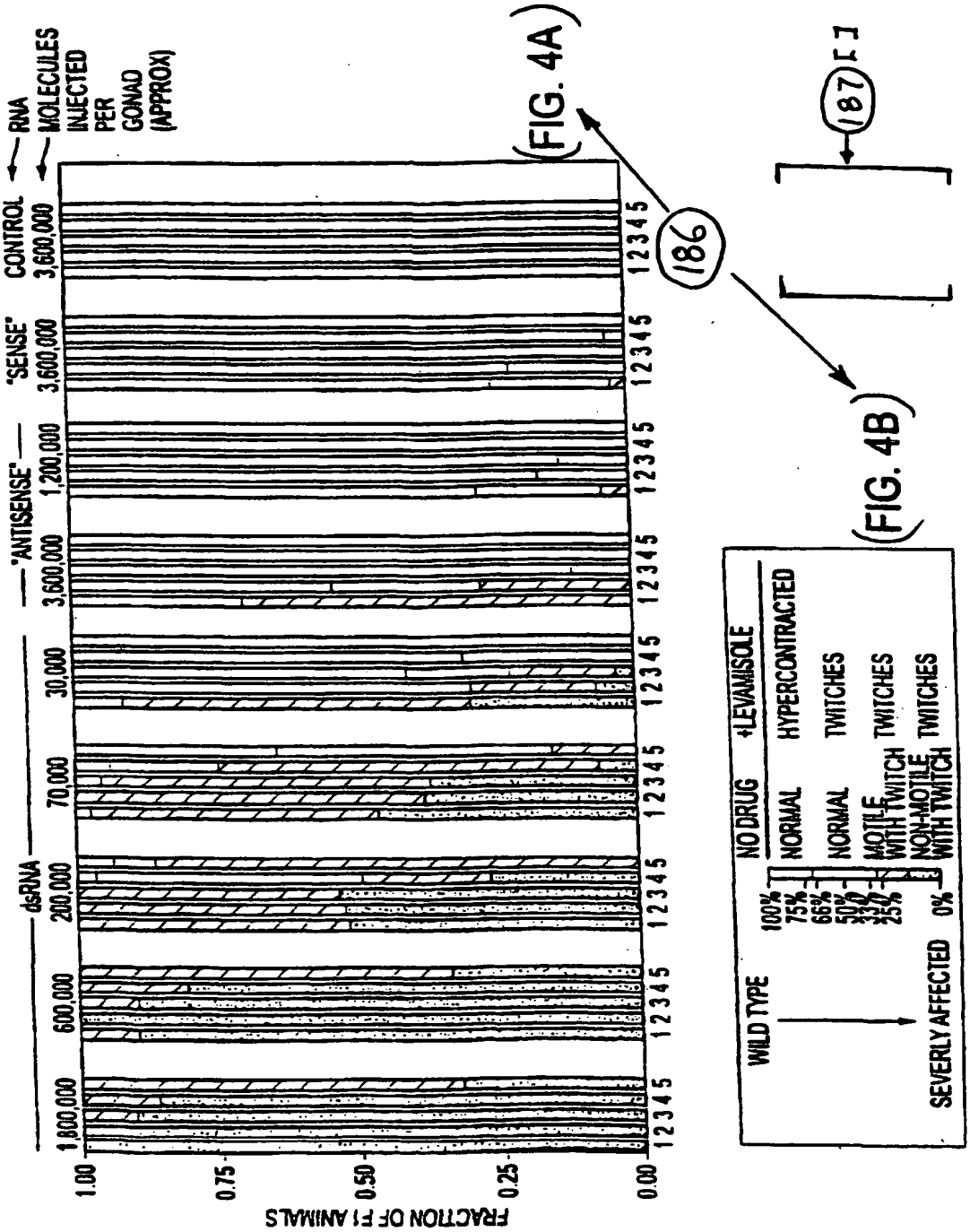



FIG. 3A FIG. 3B

FIG. 3C FIG. 3D


RNA MOLECULES INJECTED PER GONAD (APPROX)
CONTROL
"SENSE"
"ANTISENSE"
dsRNA
1,800,000
600,000
200,000
70,000
30,000
3,600,000
1,200,000
3,600,000
3,600,000
12345
FRACTION OF F1 ANIMALS
1.00
0.75
0.50
0.25
0.00
186
187
FIG. 4A
FIG. 4B
NO DRUG
+LEVAMISOLE
WILD TYPE
100%
75%
66%
50%
33%
25%
0%
SEVERLY AFFECTED
NORMAL
HYPERCONTRACTED
NORMAL
TWITCHES
MOTILE
WITH TWITCH
TWITCHES
NON-MOTILE
WITH TWITCH
TWITCHES

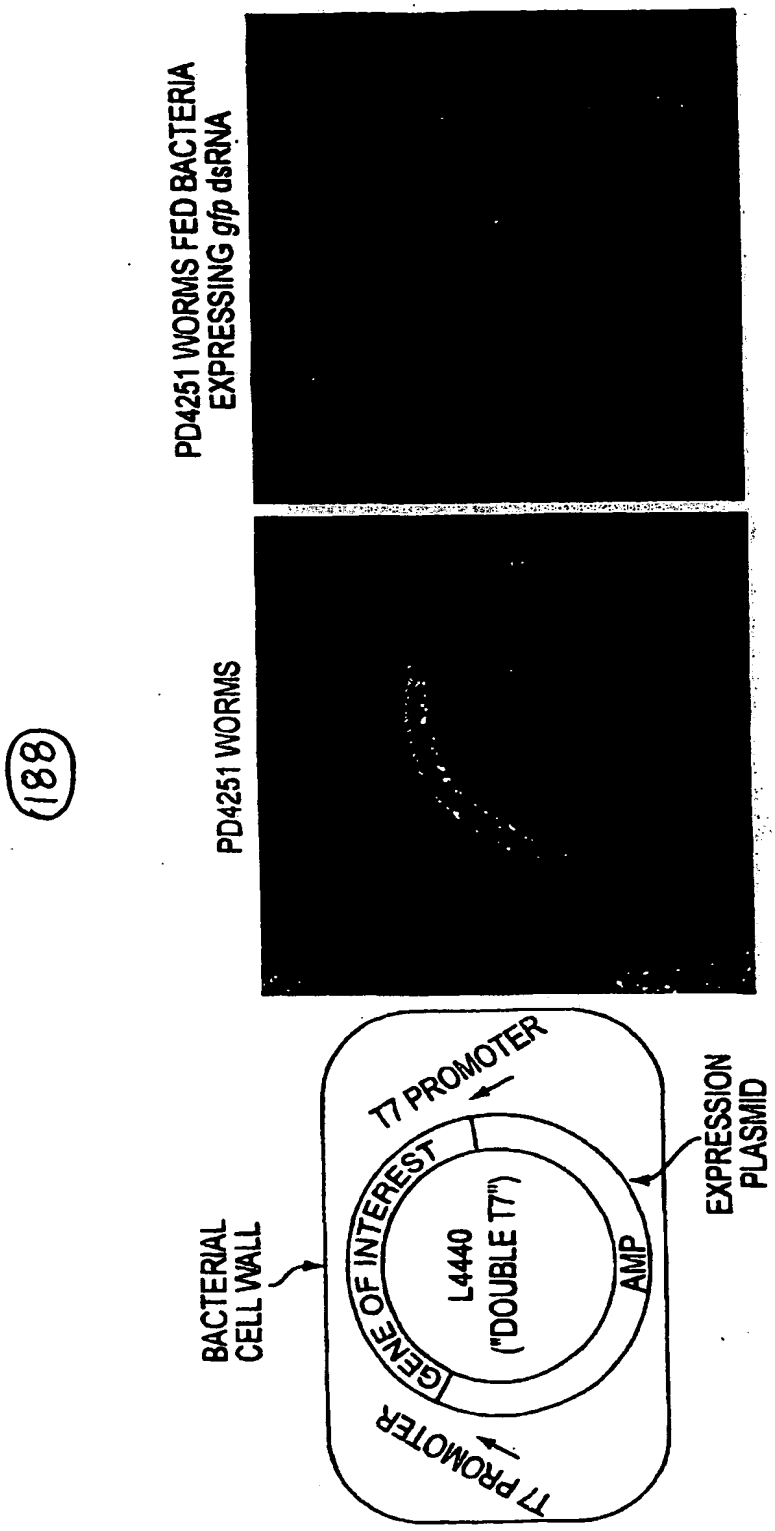



FIG. 5C

FIG. 5B

FIG. 5A

# GENETIC INHIBITION BY DOUBLE-STRANDED RNA

## RELATED APPLICATION

This application claims the benefit of U.S. Provisional Appln. No. 60/068,562, filed Dec. 23, 1997. +gi

## GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a directed change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based for example cases in which is important to produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

### Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

### Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule in vitro under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use in vivo has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells in vivo. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

### Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and Drosophila. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been hypothesized to occur; an as-yet-unidentified mechanism would then lead to de novo methylation and subsequent suppression of gene expression.

Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

### Distinction Between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

### Distinction Between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition in *C. elegans* is mediated by a triple-strand structure.

### Distinction Between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and Drosophila indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

## SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For

transcription from a transgene in vivo or an expression construct a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to in vitro use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]).

FIGS. 2A–I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-unc22A). Panels D–F show progeny of animals injected with ds-gfpG. Panels G–I demonstrate specificity. Animals are injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

FIGS. 3A–D show effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA. Micrographs show in situ hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous mex-3 RNA[20]). Panel C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos and the parent animals retain the mex-3 mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to mex-3B; no mex-3 RNA was detected. Scale: each embryo is approximately 50 μm in length.

FIG. 4 shows inhibitory activity of unc-22A as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0–6 hours, 2 for 6–15 hours, 3 for 15–27 hours, 4 for 27–41 hours, and 5 for 41–56 hours. The bottom-left diagram shows genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[8,3].

FIGS. 5A–C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole

organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxy-acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotides. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12–16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases. As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis, field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and

acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see, for example, WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology. Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells in vitro or ex vivo and then subsequently placed into an animal to affect therapy, or directly treated by in vivo administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, diges-

tive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized (or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in Index of plant Diseases in the United States (U.S. Dept. of Agriculture Handbook No. 165, 1960); Distribution of Plant-Parasitic Nematode Species in North America (Society of Nematologists, 1985); and Fungi on Plants and Plant Products in the United States (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The case with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones

of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

## Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene [1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans* (RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in C. elegans, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of cosuppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give

effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for unc-22 and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific in vitro promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters in vivo, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

## Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The unc-22 gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between unc-22 gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. unc-22 encodes an abundant but non-essential myofilament protein[7-9]. unc-22 mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of unc-22 had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (FIG. 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (FIG. 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. unc-22 expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to unc-22 did not potentiate the ability of unc-22 single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in cis to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded unc-22 segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by unc-22 dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic unc-22 loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (FIG. 1 and Table 1). unc-54 encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], fem-1 encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and hlh-1 encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment unc54C, which led to an embryonic and larval arrest phenotype not seen with unc-54 null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The unc54C segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The unc-54 and hlh-1 muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to gfp produced dramatic decreases in the fraction of fluorescent cells (FIG. 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with gfp inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived

striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13–14 versus 8–9 for embryonic muscles[18,19]). At high concentrations of gfp dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or postembryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produces a dramatic decrease in the level of the endogenous mRNA transcript (FIG. 3). Here, a mex-3 transcript that is abundant in the gonad and early embryos[20] was targeted where straightforward in situ hybridization can be performed[9]. No endogenous mex-3 mRNA was observed in animals injected with a dsRNA segment derived from mex-3 (FIG. 3D), but injection of purified mex-3 antisense RNA resulted in animals that retained substantial endogenous mRNA levels (FIG. 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for unc-22, gfp, or lacZ into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see FIG. 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals with dsRNA.

The *C. elegans* gene unc-22 encodes an abundant muscle filament protein. unc-22 null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from unc-22, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the unc-22 gene. The *C. elegans* fem-1 gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to fem-1, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a gfp transgene were fed bacteria expressing dsRNA corresponding to the gfp reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see FIG. 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from fem-1 and gfp produced no twitching, dsRNAs from unc-22 and fem-1 did not reduce gfp expression, and dsRNAs from gfp and unc-22 did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either gfp or unc-22 caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The unc-22 dsRNA was segment ds-unc22A from FIG. 1. pos-1 and sqt-3 dsRNAs were from the full length cDNA clones. pos-1 encodes an essential maternally provided component required early in embyogenesis. Mutations removing pos-1 activity have an early embryonic arrest characteristic of skn-like mutations[29,30]. Cloning and activity patterns for sqt-3 have been described[31]. *C. elegans* sqt-3 mutants have mutations in the col-1 collagen gene[31]. Phenotypes of affected animals are noted. Incidences of clear phenotypic effects in these experiments were 5–10% for unc-22, 50% for pos-1, and 5% for sqt-3. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that unc-22 dsRNA produced only Unc-22 phenotypes, pos-1 dsRNA produced only Pos-1 phenotypes, and sqt-3 dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806–811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M. K. & Fire, A. Trends in Genetics, 14, 255–258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

## Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[9], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless,

RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68° C. to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37° C. for 10–30 minutes. Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA in vitro. Non-annealed sense+antisense RNAs for unc22B and gfpG were tested for inhibitory effect and found to be much more active than the individual single strands, but 2–4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for unc22A, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of $0.5\times10^6$ to $1.0\times10^6$ molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with gfp[27] and lacZ activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (myo-3 promoter driving mitochondrially targeted GFP), pSAK2 (myo-3 promoter driving a nuclear targeted GFP-LacZ fusion), and a dpy-20 subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20–100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

FIG. 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., unc22C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (FIG. 2, panels A–H). These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-unc22A RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appear identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP) in young larva (FIG. 2A), adult (FIG. 2B), and adult body wall at high magnification (FIG. 2C).

In contrast, the progeny of animals injected with ds-gfpG RNA are affected (FIGS. 2D–F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (FIG. 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (FIG. 2E). Rare GFP positive body wall muscle cells were also seen in adult animals (two active cells are shown in FIG. 2F). Inhibition was target specific (FIGS. 2G–I). Animals were injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in FIG. 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (FIG. 2H). Scale bars in FIG. 2 are 20 μm.

The effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA was shown by in situ hybridization to embryos (FIG. 3, panels A–D). The 1262 nt mex-3 cDNA clone[20] was divided into two segments, mex-3A and mex3B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. mex-3B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and in situ hybridization (see

Reference 5). The mex-3B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to mex-3A were used to assay distribution of the endogenous mex-3 mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (FIG. 3A). Embryos from uninjected parents showed a normal pattern of endogenous mex-3 RNA (FIG. 3B). The observed pattern of mex-3 RNA was as previously described in Reference 20. Injection of purified mex-3B antisense RNA produced at most a modest effect: the resulting embryos retained mex-3 mRNA, although levels may have been somewhat less than wild type (FIG. 3C). In contrast, no mex-3 RNA was detected in embryos from parents injected with dsRNA corresponding to mex-3 (FIG. 3D). The scale of FIG. 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by unc-22A RNA was measured as a function of RNA structure and concentration (FIG. 4). Purified antisense and sense RNA from unc22A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (gfpG). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[28,29].

FIGS. 5A–C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (FIG. 5A). A bacterial strain (BL21/DE3)[29] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (FIG. 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (FIG. 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either unc-22 or gfp. This was comparably effective.

All (references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are) incorporated herein in their entirety.

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TABLE 1

Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

| Gene and Segment | | Size | Injected RNA | F1 Phenotype |
|---|---|---|---|---|
| unc-22 | | | | unc-22 null mutants: strong twitchers[7,8] |
| unc22A[a] | exon 21–22 | 742 | sense | wild type |
| | | | antisense | wild type |
| | | | sense + antisense | strong twitchers (100%) |
| unc22B | exon27 | 1033 | sense | wild type |
| | | | antisense | wild type |

TABLE 1-continued

| Gene and Segment | | Size | Injected RNA | Effects of sense, antisense, and mixed RNAs on progeny of injected animals. F1 Phenotype |
|---|---|---|---|---|
| | | | sense + antisense | strong twitchers (100%) |
| unc22C | exon 21–22[b] | 785 | sense + antisense | strong twitchers (100%) |
| fem-1 | | | | fem-1 null mutants: female (no sperm)[13] |
| fem1A | exon 10[c] | 531 | sense | hermaphrodite (98%) |
| | | | antisense | hermaphrodite (>98%) |
| | | | sense + antisense | female (72%) |
| fem1B | intron 8 | 556 | sense + antisense | hermaphrodite (>98%) |
| unc-54 | | | | unc-54 null mutants: paralyzed[7,11] |
| unc54A | exon 6 | 576 | sense | wild type (100%) |
| | | | antisense | wild type (100%) |
| | | | sense + antisense | paralyzed (100%)[d] |
| unc54B | exon 6 | 651 | sense | wild type (100%) |
| | | | antisense | wild type (100%) |
| | | | sense + antisense | paralyzed (100%)[d] |
| unc54C | exon 1–5 | 1015 | sense + antisense | arrested embryos and larvae (100%) |
| unc54D | promoter | 567 | sense + antisense | wild type (100%) |
| unc54E | intron 1 | 369 | sense + antisense | wild type (100%) |
| unc54F | intron 3 | 386 | sense + antisense | wild type (100%) |
| hlh-1 | | | | hlh-1 null mutants: lumpy-dumpy larvae[16] |
| hlh1A | exons 1–6 | 1033 | sense | wild type (<2% lpy-dpy) |
| | | | antisense | wild type (<2% lpy-dpy) |
| | | | sense + antisense | lpy-dpy larvae (>90%)[e] |
| hlh1B | exons 1–2 | 438 | sense + antisense | lpy-dpy larvae (>80%)[e] |
| hlh1C | exons 4–6 | 299 | sense + antisense | lpy-dpy larvae (>80%)[e] |
| hlh1D | intron 1 | 697 | sense + antisense | wild type (<2% lpy-dpy) |
| myo-3 driven GFP transgenes[f] | | | | makes nuclear GFP in body muscle |
| myo-3::NLS::gfp::lacZ | | | | |
| gfpG | exons 2–5 | 730 | sense | nuclear GFP-LacZ pattern of parent strain |
| | | | antisense | nuclear GFP-LacZ pattern of parent strain |
| | | | sense + antisense | nuclear GFP-LacZ absent in 98% of cells |
| lacZL | exon 12–14 | 830 | sense + antisense | nuclear GFP-LacZ absent in >95% of cells |
| myo-3::MtLS::gfp | | | | makes mitochondrial GFP in body muscle |
| gfpG | exons 2–5 | 730 | sense | mitochondrial GFP pattern of parent strain |
| | | | antisense | mitochondrial GFP pattern of parent strain |
| | | | sense + antisense | mitochondrial GFP absent in 98% of cells |
| lacZL | exon 12–14 | 830 | sense + antisense | mitochondrial GFP pattern of parent strain |

Legend of Table 1

Each RNA was injected into 6–10 adult hermaphrodites ($0.5–1\times10^6$ molecules into each gonad arm). After 4–6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20–22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12–24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each unc22A RNA preparation at a series of different concentrations. At the highest dose tested ($3.6\times10^6$ molecules per gonad), the individual sense and antisense unc22A preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-unc22A RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-unc22A RNA produced visible twitching in 30% of progeny.

b: unc22C also carries the intervening intron (43 nt).

c: fem1A also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (a1C) at 4–24 hours after injection) showed movement defects indistinguishable from those of null mutants in unc-54. A variable fraction of these animals (25–75%) failed to lay eggs (another phenotype of unc-54 null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of unc-54 activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of hlh-1 inhibitory RNA include arrested embryos and partially elongated L1 larvae (the hlh-1 null phenotype) seen in virtually all progeny from injection of ds-hlh1A and about half of the affected animals from ds-hlh1B and ds-hlh1C) and a set of less severe defects (seen with the remainder of the animals from ds-hlh1B and ds-hlh1C). The less severe phenotypes are characteristic of partial loss of function for hlh-1.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of gfp (loss of all fluorescence) and lacZ (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-gfpG caused a loss of GFP in all but 0–3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0–5 additional bodywall muscles and in the eight vulval muscles.

TABLE 2

Effect of injection point on genetic inhibition in injected animals and their progeny

| dsRNA | Site of injection | Injected animal phenotype | Progeny Phenotype |
|---|---|---|---|
| None | gonad or body cavity | no twitching | no twitching |
| None | gonad or body cavity | strong nuclear & mitochondrial GFP | strong nuclear & mitochondrial GFP |
| unc22B | Gonad | weak twitchers | strong twitchers |
| unc22B | Body Cavity Head | weak twitchers | strong twitchers |
| unc22B | Body Cavity Tail | weak twitchers | strong twitchers |
| gfpG | Gonad | lower nuclear & mitochondrial GFP | rare or absent nuclear & mitochondrial GFP |
| gfpG | Body Cavity Tail | lower nuclear & mitochondrial GFP | rare or absent nuclear & mitochondrial GFP |
| lacZL | Gonad | lower nuclear GFP | rare or absent nuclear GFP |
| lacZL | Body Cavity Tail | lower nuclear GFP | rare or absent nuclear GFP |

TABLE 3

*C. elegans* can respond in a gene-specific manner to environmental dsRNA.

| Bacterial Food | Movement | Germline Phenotype | GFP-Transgene Expression |
|---|---|---|---|
| BL21(DE3) | 0% twitch | <1% female | <1% faint GFP |
| BL21(DE3) [fem-1 dsRNA] | 0% twitch | 43% female | <1% faint GFP |
| BL21(DE3) [unc22 dsRNA] | 85% twitch | <1% female | <1% faint GFP |
| BL21(DE3) [gfp dsRNA] | 0% twitch | <1% female | 12% faint GFP |

TABLE 4

Effects of bathing *C. elegans* in a solution containing dsRNA.

| dsRNA | Biological Effect |
|---|---|
| unc-22 | Twitching (similar to partial loss of unc-22 function) |
| pos-1 | Embryonic arrest (similar to loss of pos-1 function) |
| sqt-3 | Shortened body (Dpy) (similar to partial loss of sqt-3 function) |

In Table 2 gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with gfp (fainter overall fluorescence), lacZ (loss of nuclear fluorescence), and unc-22 (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-unc22B injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-unc22A. Injections of ds-gfpG or ds-lacZL produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-gfpG and ds-lacZL produced no twitching, while injections of ds-unc22A produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

We claim:

1. A method to inhibit expression of a target gene in a cell (in vitro) comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the first ribonucleotide sequence comprises at least 25 bases which correspond to the target gene and the second ribonucleotide sequence comprises at least 25 bases which are complementary to the nucleotide sequence of the target gene.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. A method to inhibit expression of a target gene in an invertebrate organism comprising:

(a) providing an invertebrate organism containing a target cell, wherein the target cell contains the target gene and the target cell is susceptible to RNA interference, and the target gene is expressed in the target cell;

(b) contacting said invertebrate organism with a ribonucleic acid (RNA), wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide sequences are separate complementary strands that hybridize to each other to form the double-stranded molecule; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

**13**. The method of claim **12** in which the organism is a nematode.

**14**. The method of claim **13** in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

**15**. The method of claim **12** in which said double-stranded ribonucleic acid structure is at least 25 bases in length and each of the ribonucleic acid strands is able to specifically hybridize to a deoxyribonucleic acid strand of the target gene over the at least 25 bases.

**16**. The method of claim **12** in which the expression of the target gene is inhibited by at least 10%.

**17**. The method of claim **12** in which the RNA is introduced within a body cavity of the organism and outside the target cell.

**18**. The method of claim **12** in which the RNA is introduced by extracellular injection into the organism.

**19**. The method of claim **12** in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

**20**. The method of claim **19** in which the food comprises a genetically-engineered host transcribing the RNA.

**21**. The method of claim **12** in which at least one strand of the RNA is produced by transcription of an expression construct.

**22**. The method of claim **21** in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

# CERTIFICATE OF CORRECTION

PATENT NO. : 6,506,559 B1
DATED : January 14, 2003
INVENTOR(S) : Andrew Fire et al.



It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73] Assignee, should read:
-- [73] Assignee: **The Carnegie Institution of Washington**, Washington, DC (US); **The University of Massachusetts**, Boston, Massachusetts (US) --

Signed and Sealed this

Sixteenth Day of September, 2003



JAMES E. ROGAN
*Director of the United States Patent and Trademark Office*

**Differences Between U.S. Patent No. 6,506,559 B1, issued January 14, 2003 ("Fire et al. Patent") and U.S. Provisional Application No. 60/068,562, filed December 23, 1997 ("Fire et al. Provisional")**

- ( ) indicates text which appears only in Fire et al. Patent
- [ ] indicates text which appears only in Fire et al. Provisional
- In Provisional is ". . ." indicates text found in both Fire et al. Patent and Fire et al. Provisional, but with changes in Fire et al. Patent.
- { } gives more specific location of the text in the Provisional, where the text in the Fire et al. Patent does not follow the same order as the Fire et al. Provisional.

1. In Provisional is ":"
2. In Provisional is ","
3. Provisional only

   [, presented in the literature over the last few month,]
4. In Provisional is ". However"
5. Patent only

   (been used to)
6. In Provisional is "inhibited"
7. Patent only

   (The extreme . . . formation)
8. Provisional only

   [These distinctions]
9. Provisional only

   [and non-replicating]
10. Provisional only

    [The process may be practice *ex vivo* or *in vivo*.]

11. Provisional only

[(i.e., a cellular gene)]

12. Provisional only

[(i.e., a cellular gene present in the genome)]

13. Provisional only

[(i.e., a gene construct inserted at an ectopic site in the genome of the cell)]

14. Provisional only

[viral]

15. Patent only

(of a pathogen)

16. Provisional only

[(e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells)]

17. Provisional only

[target gene transcription,]

18. Provisional only

[,]

19. In Provisional is "multiple"

20. Provisional only

[Fully duplex RNA lacks an unpaired region with single-stranded structure.]

21. Provisional only

[(e.g., at least 5, 10, 100, or 1000 copies per cell)]

22. In Provisional is "are"

23. Provisional only

[(see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein)]

24. Provisional only

    [Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and target gene is preferred for the identical nucleotide sequences.]

25. Provisional only

    [(e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hr). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200 or 400 nucleotides]

26. Patent only

    (protozoan . . . or)

27. Provisional only

    [or yeast]

28. In Provisional is "vector"

29. Provisional only

    [(e.g., promoter, enhancer, silencer)]

30. In Provisional is "is"

31. In Provisional is "or"

32. Patent only

    (, introduced . . . affected)

33. In Provisional is "are preferred such as"

34. Patent only

    (directly)

35. In Provisional is "of a solution containing the RNA."

36. Patent only

    (present)

37. In Provisional is "that is"

38. In Provisional is "The present"

39. In Provisional is "the present"

40. In Provisional is "The noted disadvantages"

41. Patent only

(the)

42. Provisional only

[These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for RNAi is designated with the name of the gene followed by a single letter (e.g., *unc22*C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.]

43. In Provisional is "H"

44. In Provisional is ":"

45. Provisional only

[, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene expression]

46. Patent only

(visualized . . . microscope)

47. Provisional only

[These GFP patterns appear identical to the parent strain, with prominent fluorescence in nuclei (the

nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP).]

48. Provisional only

[Observable GFP fluorescence is completely absent in over 95% of cells. Only a single active cell is seen in the larva in panel D, while the adult animal in panel E shows staining in none of the striated body wall muscles. Inhibition is not effective in all tissues: the entire vulval musculature expresses active GFP in the adult animal shown in panel E. Panel F shows two rare GFP positive cells in an adult. Both cells express both nuclear-targeted GFP-LacZ and mitochondrial GFP.]

49. In Provisional is ": animals"

50. Provisional only

[In the animals derived from this injection, mitochondrial-targeted GFP appears unaffected while the nuclear-targeted GFP-LacZ is absent from almost all cells (e.g. larva in panel G).]

51. Patent only

((dark stain))

52. Provisional only

[The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 hours before fixation and in situ hybridization (see reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while

>90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos are shown; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults.]

53. In Provisional is "The scale is such that"

54. Provisional only

[Purified antisense and sense RNA from *unc22*A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6, 15, 27, 41 and 56 hours after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole.]

55. Provisional only

[in the graph]

56. Patent only

(Progeny . . . 56 hours The)

57. Provisional only

[:]

58. In Provisional is "showing"

59. Patent only

(FIGs. 5A-C . . . gfp.)

60. In Provisional is "DESCRIPTION OF PREFERRED EMBODIMENTS"

61. Patent only

(producing)

62. Patent only

(by introducing)

63. Provisional only

[with]

64. Provisional only

[¶], *i.e.* a paragraph break

65. Patent only, *but see* Provisional {page 5, line 28 to page 6, line 12}

65.1. Provisional only

[or into the extracellular environment] {page 6, line 1}

65.2. Provisional only

[The process may be practiced ex vivo or in vivo.] {page 6, line 3}

65.3. In Provisional is "viral gene which is present in the cell after infection thereof." {page 6, line 8-9}

65.4. In Provisional is "the procedure" {page 6, line 10}

66. Provisional only

[Sequence]

67. Patent only

(nuclease protection,)

68. Patent only

(gene . . . microarray,)

69. Patent only

((ELISA))

70. Patent only

((RIA), other immunoassays)

71. Patent only

((FACS))

72. Patent only

(acetohydroxyacid synthase (AHAS),)

73. Patent only

((AP))

74. Patent only

((GUS))

75. Patent only

((CAT))

76. Patent only

(horseradish peroxidase (HRP))

77. Patent only

((Luc), . . . (OCS),)

78. In Provisional is "or"

79. Patent only

(Multiple . . . tetracyclin.)

80. Patent only

(as compared . . . invention)

81. Patent only, *but see* Provisional {page 6, line 12 to page 7, line 9}

81.1. Provisional only

[target gene transcription] {page 6, lines 15-16)

81.2. Patent only

(As an example . . . region.)

81.3. In Provisional is "; it" {page 6, line 17}

81.4. Patent only

(For example . . . synthesis.)

81.5. In Provisional is "multiple" {page 6, line 20}

81.6. Provisional only
[Fully duplex RNA lacks an unpaired region with single stranded structure.] {page 6, lines 20 - 21}

81.7. Patent only
(500)

81.8. Patent only
(; lower . . . applications)

81.9. Patent only
(sequence comparison and)

81.10. Patent only
(by, for example, . . . Group).)

81.11. Patent only
(the portion of)

81.12. Provisional only
[for the identical nucleotide sequences] {page 7, line 4}

81.13. Patent only
(hybridization)

81.14. Patent only
(; followed by washing)

81.15. Patent only
(300)

82. Patent only
(As disclose . . . divergence.)

83. Patent only
(protozoan . . . or)

84. Provisional only
[or yeast]

85. Patent only

(Preferred . . . morphologies.)

86. Patent only

(; field . . . bean)

87. Patent only

(cotton . . . sorghum)

88. Patent only

(sunflower,)

89. Patent only

(; vegetable . . . carrot,)

90. Patent only

(celery . . . pepper,)

91. Patent only

(pumpkin . . . juniper;)

92. Patent only

(palm, poplar,)

93. Patent only

(redwood . . . and)

94. In Provisional is "cow"

95. Patent only

(sheep)

96. Patent only

(Representative . . . Homoptera.)

97. In Provisional is "That"

98. Patent only

(having the target gene)

99. Patent only, *but see* Provisional {page 7, line 11 to page 7 line 15}

99.1. In Provisional is "vector" {page 7, line 14}

99.2. Patent only

(, splice . . . polyadenylation)

99.3. In Provisional is "is" {page 7, line 15}

100. Patent only

(Inhibition . . . apparatus.)

101. Patent only

(or enzymatically)

102. Patent only

(a cellular)

103. Provisional only

[of the cell]

104. In Provisional is "construction"

105. In Provisional is "vector"

106. Patent only

([32, 33, 34])

107. Provisional only

[Goeddel, *Gene Expression Technology*, Academic Press, 1990; Kreigler, *Gene Transfer and Expression*, Stockton Press, 1990; Murray, *Gene Transfer and Expression Protocols*, Humana Press, 1991;]

108. Patent only

(also)

109. Patent only

(U.S. Pat. . . . 5,804,693;)

110. In Provisional is "Preferably"

111. In Provisional is "is"

112. Patent only

(and/or . . . strands)

113. Provisional only

[The]

114. In Provisional is "introduced directly"

115. Patent only

(introduced)

116. Patent only

(into)

117. Provisional only

[Examples of extracellular spaces into which the RNA may be introduced include]

118. In Provisional is "or"

119. Patent only

(introduced orally . . . may also be used.)

120. Patent only

(the roots,)

121. Patent only

(the)

122. Patent only

(A transgenic . . . organism.)

123. In Provisional is "are preferred such as for example,"

124. In Provisional is "with"

125. In Provisional is "vector"

126. In Provisional is "vector"

127. In Provisional is "vector"

128. Patent only

(Other methods . . . target gene.)

129. Patent only

(a cell . . . introduced into)

130. Patent only

(carcinogenic/tumorigenic)

131. Patent only

(or maintenance)

132. Patent only

(Treatment . . . envisioned.)

133. Patent only

(or transformed . . . in *C. elegans*)

134. In Provisional is "is"

135. Provisional only

[¶], *i.e.* a paragraph break

136. In Provisional is "can be used"

137. In Provisional is "are"

138. In Provisional is "For instance,"

139. Patent only

(RNA-mediated mechanism was)

140. Patent only

(RNAi)

141. In Provisional is "has"

142. In Provisional is "In particular"

143. In Provisional is "co-suppression"

144. Patent only

(crude)

145. In Provisional is "We"

146. Provisional only

[(see Figure 4).]

147. In Provisional is "interference"

148. Patent only

(While . . . activity)

149. In Provisional is "interfering"

150. In Provisional is "We"

151. Provisional only

[the]

152. Patent only

(of these crude preparations)

153. Provisional only

[below]

154. In Provisional is "and that this subpopulation was"

155. Provisional only

[We disclose that the non-purified RNA populations that were effective in inhibition assays herein include some molecules with double-stranded character.]

156. In Provisional is "we"

157. Patent only

(Analysis . . . Genes)

158. Patent only, *but see* Provisional {page 17, line 3 to page 20, line 23}

158.1. In Provisional is "interference" {page 17, line 11}

158.2. In Provisional is "Table 1" {page 17, line 12}

158.3. In Provisional is "-" {page 17, line 12}

158.4. In Provisional is "-" {page 17, line 15}

158.5. In Provisional is "-" {page 17, line 19}

158.6. In Provisional is "our" {page 18, line 3}

158.7. In Provisional is "-" {page 18, line 3}

158.8. In Provisional is "We found this not" {page 18, line 4}

158.9. Provisional only

[We] {page 18, line 6}

158.10. Patent only

(was)

158.11. In Provisional is "interference" {page 18, line 7}

158.12. In Provisional is "interference" {page 18, line 10}

158.13. Provisional only

[we have only observed] {page 18, line 10}

158.14. In Provisional is "interference" {page 18, line 10}

158.15. Patent only

(was observed only)

158.16. Provisional only

[We assessed] {page 18, line 14}

158.17. In Provisional is "interfere with" {page 18, line 26}

158.18. Patent only

(the)

158.19. Provisional only

[we used] {page 19, line 5}

158.20. Patent only

(was used)

158.21. In Provisional is "we" {page 19, line 11}

158.22. Provisional only

[we saw] {page 19, line 17}

158.23. Patent only

(was noted)

158.24. Provisional only

[we found that] {page 20, line 3}

158.25. In Provisional is "produces" {page 20, line 3}

158.26. Provisional only

[we targeted] {page 20, line 4}

158.27. Patent only
(was targeted)

158.28. Provisional only
[5] {page 20, line 6}

158.29. In Provisional is ". In contrast," {page 20, line 7}

158.30. In Provisional is "D" {page 20, line 9}

158.31. Patent only
(Table 3 . . . 1998.)

158.32. In Provisional is "we" {page 20, line 22}

158.33. Provisional only
[one case] {page 20, line 22}

158.34. Provisional only
[This would not be a consideration for a target gene present in a single copy in the genome.] {page 20, lines 23-24}

159. Patent only
(of)

160. In Provisional is "interference"

161. In Provisional is "interference"

162. Patent only
(Following . . . structure,)

163. In Provisional is "/"

164. In Provisional is "RNA"

165. Patent only
(Methods for)

166. Patent only
(Additional Description of the Results)

167. Patent only, *but see* Provisional {page 8, line 10 to page 10, line 12}

167.1. Patent only

(sequence . . . follows:)

167.2. In Provisional is "RNAi" {page 8, line 15}

167.3. In Provisional is "Figure 2A-H show analysis" {page 8, line 21}

167.4. Patent only

(were analyzed)

167.5. Provisional only

[effects] {page 8, line 21}

167.6. Patent only

((FIG. 2, panels A-H))

167.7. Patent only

(ds-unc22A . . . affected.)

167.8. Provisional only

[The micrographs show progeny of injected animals. Panel A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (*ds-unc22*A).] {page 8, lines 26-28}

167.9. In Provisional is "These" {page 8, line 28}

167.10. Patent only

(of the progeny)

167.11. In Provisional is "appear" {page 8, line 28}

167.12. Patent only

(: young larva . . .(FIG. 2C).)

167.13. Patent only

(In contrast, the)

167.14. Provisional only

[Panels D-F show] {page 9, line 1}

167.15. Patent only

(RNA . . . (FIGS. 2D-F).)

167.16. Patent only

(Few . . . muscle cells).)

167.17. Provisional only

[Only a single active cell is seen in the larva in panel D, while the adult animal in panel E shows staining in none of the striated body wall muscles.] {page 9, lines 3-4}

167.18. In Provisional is "is" {page 9, line 4}

167.19. In Provisional is "expresses" {page 9, line 5}

167.20. In Provisional is "shown in panel E." {page 9, line 6}

167.21. Provisional only

[Panel F shows two] {page 9, line 6}

167.22. Patent only

(were also seen)

167.23. In Provisional is "in an" {page 9, line 6}

167.24. Patent only

(animals . . . FIG 2F).)

167.25. In Provisional is "Panels G-I demonstrate specificity:" {page 9, line 7}

167.26. In Provisional is "are" {page 9, line 8}

167.27. In Provisional is "is" {page 9, line 11}

167.28. Provisional only

[e.g.,] {page 9, line 11}

167.29. In Provisional is "Panel H shows a typical adult, with" {page 9, line 11}

167.30. Provisional only
[lacking] {page 9, line 12}

167.31. Patent only
(activity)

167.32. Patent only
((FIG. 2H))

167.33. Patent only
(in FIG. 2)

167.34. Provisional only
[Figures 3 A-D show] {page 9, line 14}

167.35. Patent only
(The)

167.36. Patent only
(was show . . . panels A-D).)

167.37. Provisional only
[Micrographs show *in situ* hybridization to embryos.] {page 9, line 15}

167.38. In Provisional is "fed" {page 9, line 19}

167.39. In Provisional is "are shown;" {page 9, line 23}

167.40. In Provisional is "Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous mex-3 $RNA^{20}$). Panel C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos and the parent animals retain the mex-3 mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with

dsRNA corresponding to mex-3B; no mex-3 RNA was detected." {page 9 line 14 - page 10, line 1}

167.41. Patent only
(of FIG. 3)

167.42. In Provisional is "Figure 4 shows" {page 10, line 3}

167.43. In Provisional is "of" {page 10, line 3}

167.44. Patent only
(was measured)

167.45. Patent only
(RNA)

167.46. Patent only
((FIG. 4))

167.47. In Provisional is "6, 15, 27, 41, and 56 hours" {page 10, line 6}

167.48. Patent only
(The)

167.49. Provisional only
[:] {page 10, line 10}

167.50. In Provisional is "showing" {page 10, line 10}

167.51. Patent only
(the)

168. Patent only
(FIG. 5A-C . . . effective.)

169. Patent only, *but see* Provisional {page 20, line 25 to page 20, line 25}

169.1. Patent only
(references (e.g.,)

169.2. Patent only

(application,)

169.3. Patent only

(indicative . . . disclosures are)

170. In provisional is "Reference"

171. In Provisional is "Karn"

172. Patent only

(28 . . . 1991.)

173. In Provisional is "layed" {page 24, line 10}

174. Patent only

(In Table 2,)

175. Provisional only

[present] {page 26, line 22}

176. Patent only

(New Table 3 added)

177. Patent only

(in vitro)

178. In Provisional is "has" {page 27, line 5}

179. Provisional only

[structure with an identical nucleotide sequence as compared to a portion of the target gene.] {page 27, lines 5-6}

180. Patent only

(molecule . . . target gene.)

181. Provisional only

[identical nucleotide sequence is at least 50 nucleotides in length.] {page 27, lines 24-25}

182. Patent only

(first ribonucleotide . . . target gene.)

183. In Provisional is

"12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of any one of claims 1-12 in which the RNA has one self-complementary strand.

14. The method of any one of claims 1-12 in which the RNA has two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of any one of claims 1-12 in which the RNA has no single stranded structure.

18. The method of any one of claims 1-12 in which the RNA is introduced within the body cavity of an animal and outside the cell.

19. The method of any one of claims 1-12 in which the RNA is introduced by extracellular injection into a body cavity of an organism.

20. The method of any one of claims 1-12 in which an expression vector in a cell produces the RNA." {page 28, lines 1-26}

184. Patent only

(Labels "FIG. 2", "CONTROL RNA (ds-*unc22a*)", "ds-*gfpG* RNA", "ds-*laxZL* RNA", "L1" and "ADULT" are added to the graphs in Figure 2)

185. Patent only

(Label "FIG. 3" is added to the caption for each graph in Figure 3)

186. Patent only

(Labels "FIG. 4A" and "FIG. 4B" are added)

187. Provisional only

[Section labeled "Progeny Cohort Group"]

188. Patent only

(New Figure 5 added)

# EXHIBIT E

Application of Saunders,
Cust. & Pat.App. 1971.

United States Court of Customs and Patent Appeals.
Application of James H. SAUNDERS and Paul G. Gemeinhardt.
**Patent Appeal No. 8500.**

June 24, 1971.

Proceedings on application for patent, serial No. 758,890. The Patent Office Board of Appeals affirmed primary examiner's final rejection of certain claims, and applicant appealed. The United States Court of Customs and Patent Appeals, Rich, J., held that claims 11 and 26 of application for patent for methods of preparing cellular polyurethane foam were improperly rejected as anticipated by the prior art. The Court further held that claims 6, 8-10, 19-21 and 23 were properly rejected on ground of obviousness.

Modified.

Baldwin, J., concurred and filed opinion.

West Headnotes

**[1] Patents 291 ⟸113(6)**

291 Patents
291IV Applications and Proceedings Thereon
291k113 Appeals from Decisions of Commissioner of Patents
291k113(6) k. Review on Appeal in General. Most Cited Cases
Correction in affidavit filed to rebut inference of obviousness would be considered on appeal from rejection of certain claims of alleged invention where discovery of error and correction thereof did not alter positions about issues to be decided by the court. Patent Office Practice Rules, rule 132, 35 U.S.C.A. App.

**[2] Patents 291 ⟸66(1.4)**

291 Patents
291II Patentability
291II(D) Anticipation
291k63 Prior Patents
291k66 Operation and Effect
291k66(1.4) k. Combining Prior Patents. Most Cited Cases
Where it would require combining some other part of one prior patent, not clearly referred to by another prior patent, so that the two references were being used to show obviousness to one of ordinary skill in the art, the two references together could not constitute an "anticipation" of subsequently claimed invention in a technical sense of that term in patent law. 35 U.S.C.A. § 102.

**[3] Patents 291 ⟸66(1.24)**

291 Patents
291II Patentability
291II(D) Anticipation
291k63 Prior Patents
291k66 Operation and Effect
291k66(1.24) k. Process, Method, and Apparatus Claims in General. Most Cited Cases
Claims 11 and 26 of application for patent for methods of preparing cellular polyurethane foam were improperly rejected as anticipated by the prior art. 35 U.S.C.A. § 104.

**[4] Patents 291 ⟸16.4**

291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k16.4 k. Results and Means of Producing. Most Cited Cases
(Formerly 291k18)
Claims 6, 8-10, 19-21, and 23 of application for patent for methods of preparing cellular polyurethan foam were properly rejected on ground of obviousness. 35 U.S.C.A. § 103.

**[5] Patents 291 ⟸36(1)**

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291 Patents
291II Patentability
291II(A) Invention; Obviousness
291k36 Weight and Sufficiency
291k36(1) k. In General. Most Cited Cases

**Patents 291 ⇐113(6)**

291 Patents
291IV Applications and Proceedings Thereon
291k113 Appeals from Decisions of Commissioner of Patents
291k113(6) k. Review on Appeal in General. Most Cited Cases
In those cases where the applicants have attempted to rebut a showing of the prima facie obviousness of the subject matter claimed by the introduction of objective evidence of nonobviousness, both court of customs and patent appeals and tribunals of the patent office must give full consideration to the applicants' evidence and reach a decision on the question of nonobviousness on the basis of relative strength of the applicants' showing and the prima facie case made by the patent office. 35 U.S.C.A. § 103.

**[6] Patents 291 ⇐113(6)**

291 Patents
291IV Applications and Proceedings Thereon
291k113 Appeals from Decisions of Commissioner of Patents
291k113(6) k. Review on Appeal in General. Most Cited Cases
Applicants' evidence, submitted in attempt to rebut patent office's prima facie showing of obviousness of subject matter, was not to be disregarded by court of customs and patent appeals on ground that, as result of vicissitudes of prosecution applicants were claiming much less than they originally did and that in fact, what they were subsequently claiming was originally only a preferred class within much broader claims. 35 U.S.C.A. §§ 112, 132.

**[7] Patents 291 ⇐109**

291 Patents
291IV Applications and Proceedings Thereon
291k109 k. Amendment of Application. Most Cited Cases
An applicant is not prohibited from changing what he regards as his invention during the pendency of patent application. 35 U.S.C.A. §§ 112, 132.

****600 *1317** W. Brown Morton, Jr., Martin J. Brown, Washington, D.C., and Carl G. Ries, Houston, Tex., attorneys of record, for appellants.
S. Wm. Cocharan, Washington, D.C., for Commissioner of Patents. Jack E. Armore Washington, D.C., of counsel.

Before RICH, ALMOND, BALDWIN and LANE, Judges.

RICH, Judge.
This appeal is from the decision of the Patent Office Board of Appeals affirming the examiner's rejection of claims 6, 8-11, 19-21, 23, and 26 in appellants' application serial No. 758,890, filed September 4, 1958, for methods for preparing cellular polurethane foam. We affirm in part and reverse in part.

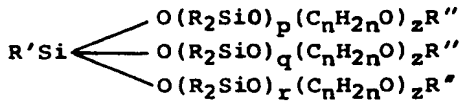
THE INVENTION

Appellants claim a family of methods for preparing cellular polyurethane foam by what may be generally characterized as the 'one shot' technique. Claim 23, which the parties agree is illustrative of the broad claims on appeal, sets forth the process adequately for present purposes (subparagraphing and emphasis of the recitation principally in controversy supplied):

23. A method for making a cellular polyurethane which comprises.

***1318** simultaneously mixing and reacting an organic polyisocyanate with a polyalkylene ether having at least 2 hydroxyl groups in the presence of a compound having the formula

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wherein

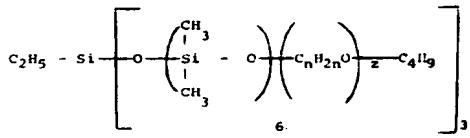
R and R' are saturated hydrocarbon radicals having from 1 to 8 carbon atoms,

R' is selected from the group consisting of hydrogen and a saturated hydrocarbon radical having from 1 to 8 carbon atoms;

p, q and r each have a value of from 2 to 15 and

(C(n)H(2n)O)(z) is a mixture of oxyethylene units and oxypropylene units containing 17 to 19 oxyethylene units and 11 to 15 oxypropylene units.

Claim 26, which the parties agree is illustrative of the narrow claims on appeal, differs from claim 23 principally in that it recites the following subgenus of the genus of silicone surfactants recited in claim 23:



in which the (C(n)H(2n)O)(z) component is a mixture of 17 oxyethylene units and 13 oxypropylene units. It also differs from claim 23 in that it calls for a tertiary amine catalyst and water, neither of which is recited in claim 23.

**601 THE REJECTION

The references relied upon are:

| | | |
|---|---|---|
| Rochow | 2,258,218 | Oct. 7, 1941 |
| Roberts et al. | 2,425,755 | Aug. 19, 1947 |
| Bailey et al. | 2,834,748 | May 13, 1958 |
| Hoppe et al. | Re.24,514 | Aug. 12, 1958 |
| Harris | 2,901,445 | Aug. 25, 1959 |
| Britain | 2,949,431 | Aug. 16, 1960 |
| Hostettler | 3,194,773 | July 13, 1965 |
| (Grandparent application Serial No. 686,009, filed Sept. 25, 1957)[1] | | |

FN1. Appellants have not questioned the availability against them of the disclosure contained in Hostettler's grandparent application. In re Lund, 376 F.2d 982, 54 CCPA 1361 (1967), nor have they argued that the disclosure therein relied upon by the Patent Office was not 'as to such invention' within the meaning of 35 U .S.C. 120.

*1319 Hostettler's grandparent application (henceforth simply 'hostettler') is the basic reference. Hostettler

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teaches a one-shot method for preparing polyurethane foams comprising reacting an organic polyisocyanate with a polyalkylene ether polyol in the presence of water, a tin catalyst, and a surfactant of the general formula shown in claim 23, but it clearly does not explicitly teach the use of such surfactants in which the (C(n)H(2n)O)(z) component 'is a mixture of oxyethylene units and oxypropylene units containing 17 to 19 oxyethylene units and 11 to 15 oxypropylene units,' much less the still smaller family of surfactants recited in claim 26. However, it was the examiner's position that, 'Since the (grand) parent application of Hostettler specifically mentions Bailey et al and because Bailey et al specifically mention Roberts et al, * * * Bailey et al and Roberts et al are incorporated into the (grand) parent application of Hostettler.'Bailey teaches how to make certain siloxane-oxyalkylene copolymers of the type Hostettler uses as surfactants, including one having approximately 17 oxyethylene units and 13 oxypropylene units, and Roberts in turn teaches how to make certain starting materials used by Bailey. Treating the three references as one, the examiner held that 'the (grand) parent application of Hostettler anticipates (35 USC 102) the claims which do not call for the presence of a catalyst.'In the alternative, the examiner held that

* * * one skilled in the art would find it obvious (35 USC 103) to review the Bailey et al and Roberts et al patent in conjunction with the disclosure of the Hostettler parent application and in making such a review would find it obvious to use as a surfactant inter alia those claimed herein since Hostettler's parent case specifically refers to Bailey et al who in turn specifically refers to Roberts et al.

The examiner held the subject matter of the claims which do call for the presence of a catalyst unpatentable under 35 U.S.C. 103 because of the Hostettler-Bailey-Roberts reference when taken in view of Hoppe, Harris, and Britain (Rochow being cited merely 'to show the state of the art'). Hoppe, Harris, and Britan all teach the use of tertiary amines as catalysts in reactions of the general type employed by appellants.

The board affirmed the rejection of the claims which do not recite the tertiary amine catalyst on the section 103 ground without reversing the section 102 ground, and on appeal the solicitor has continued to press the section 102 ground vigorously. However, the board stated that 'It is not necessary to go any further back than Bailey et al. to find the specified portions of the appealed claims,' and it ignored the Roberts reference. Both the solicitor and appellants have done likewise in their briefs, and we, too, will consider only the Hostettler and Bailey references. The board also affirmed the section 103 rejection **1320** of the claims reciting the tertiary amine catalyst on the ground that 'nothing more than the expected skill of the ordinary technican would be required to utilize the tertiary amine catalysts in **602** lieu of the tin catalyst (used by Hostettler).'

Both the examiner and the board gave consideration to the Rule 132 affidavits, filed by appellants to rebut the inference of obviousness arising from the fact that the surfactants they used were structurally very similar to the surfactants used by Hostettler, but both found them unpersuasive of nonobviousness on the grounds that (1) the affidavits compare the wrong things, (2) most of the comparisons are made in subjective terms like 'good' and 'coarse,' and (3) the objective comparisons indicated that 'other formulations changes known in the art may have as great or greater an effect than a change in surfactant.'

OPINION

Before entering upon a discussion of the substance of this case, one preliminary matter merits some attention. A week before this case was heard, counsel for appellants wrote informing us that an error had been recently (apparently, two days previously) discovered in one of the Rule 132 affidavits in this case.[FN2] According to their letter, the discovery of the error and the correction thereof did not alter appellants' positions about the issues to be decided by the court, but the error was brought to the court's attention 'in view of th duty possibly imposed on appellants by Precision (Instrument Mfg.) Company v. Automotive (Maintenance Machinery) Company, 324 U.S. 806 (65 S.Ct. 993, 89 L.Ed. 1381) (1945).' At oral argument, the solicitor conceded that the error was not material and stated that he had no objection to the court's being informed of the

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error, although he reminded us that the supplementary affidavit correcting the error was not part of the record before us.

> FN2. An affidavit which accompanied the letter explained that the affiant was also the nominal author or an affidavit in the record in this case and that, while reviewing the original notebook entries from which his previous affidavit had been prepared by appellants' then attorney, which review was made in preparation for a conference with appellants' present lawyers, he had 'noted for the first time that the references in said affidavit to stannous octoate appearing in paragraphs 7, 25, 26 Table I, and Table II thereof are erroneous in that my original notebooks show that I had used the tin catalyst identified in the notebooks as 'T-12' which was not stannous octoate.'The affidavit then goes on to explain how the error must have been made in decoding the nomenclature 'T-12' and why the affiant did not notice the error when he read the affidavit before signing it ('because I was so used to working with stannous octoate'). According to the new affidavit, the catalyst he actually used was dibutyl tin dilaurate, the same catalyst used by Hostettler.

[1] As the solicitor implied, the decision of this court in patent cases can be based only 'on the evidence produced before the Patent Office,'35 U.S.C. 144, which does not include appellants' supplementary affidavit in this case. However, counsel for both parties having**1321** agreed that the error and its correction are not material to the issues on appeal, we see no reason why we cannot take note of the correction and proceed to the resolution of those issues without remanding the case and without leaving appellants and their attorneys open to a possible future charge of having dealt with this court with less than complete candor.

I. The Section 102 Rejection

[2] The section 102 rejection in this case can be sustained only on the theory that Hostettler expressly incorporates a particular part of Bailey by reference. If it would require combining some other part of Bailey, not clearly referred to by Hostettler, so that the two references are being used to show obviousness to one of ordinary skill in the art, the two references together simply cannot constitute an 'anticipation' in the technical sense of that term in patent law. Pat.L.Persp., L.Persp., A.1(1), Dev.A.1(1)-10 (Nov. 1970).

Hostettler teaches the use of a large number of classes of siloxane surfactants, one of which differs significantly from appellants' only in that he states **603 that in the expression (C(n)H(2n)O)(z), 'n is an integer from 2 to 4 denoting the number of carbon atoms in the oxyalkylene group; and z is an integer having a value of at least 5 and denotes the length of the oxyalkylene chain.'Thus, Hostettler refers to oxyethylene (n=2), oxypropylene (n=3), and oxybutylene (n=4) but does not specifically disclose the particular ratios of oxyethylene to oxypropylene to oxybutylene groups recited in appellants' claims,[FN3] nor does it even indicate that the chains can consist of mixtures of different kinds of oxyalkylenes.

> FN3. There are no oxybutylene groups recited in the methods of the appealed claims.

However, some pages later, after setting forth a number of other surfactants, Hostettler indicates that:

The above-described siloxane-oxyalkylene block copolymers can be prepared in accordance with the procedures described and claimed in the copending application of D. L. Bailey and F. M. O'Conner, Serial No. 417,935, filed December 14, 1953.

The Bailey et al. application there referred to is now the Bailey et al. reference patent, and Example I(a) of Bailey discloses how to make a siloxane-oxyalkylene block copolymer of this type which the board found, 'On conversion to a molar basis,' to contain approximately 17 oxyethylene units and 13 oxypropylene units.

Appellants have conceded that Bailey Example I(a) 'describes a copolymer of the type claimed by appellants,' but they argue that it is not one of 'The above-described siloxane-oxyalkylene block

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*1322 referred to in Hostettler because Hostettler 'does not disclose copolymer surfactants in which the oxyalkylene groups are a mixture of oxyethylene and oxypropylene units * * *.' Therefor, appellants argue, Example I(a) is not a part of Bailey which is incorporated into Hostettler by reference. The solicitor, on the other hand, argues that Hostettler's disclosure 'that 'n' in the (C(n)H(2n)O)(z) group * * * is an integer from 2 to 4 * * * includes a mixture of oxyalkylene units.'Therefore, the solicitor argues, the surfactant copolymer in Example I(a) of Bailey is 'within the ambit' of Hostettler's surfactant formula, and Hostettler's subsequent statement that the 'above-described siloxane-oxyalkylene block copolymers can be prepared' according to the procedures of Bailey is effective to incorporate Example I(a) of Bailey into the Hostettler reference.

[3] While we must concede that the matter is not free from doubt, on balance we agree with appellants that the language 'n is an integer from 2 to 4', coupled with the reference to Bailey, would be taken by those skilled in the art only to indicate that Bailey taught how to make surfactants of this general type in which the oxyalkylene chains were composed entirely of one type of oxyalkylene and would not expressly indicate that the other compounds which Bailey also teaches how to make could also be employed as surfactants in Hostetter's process. Compare In re Lund, supra fn. 1, 376 F.2d at 988-90, 54 CCPA at 1370-72. Accordingly, we will not sustain the rejection under 35 U.S.C. 102.

II. The Section 103 Rejection

[4] However, despite the fact that we agree with appellants that the subject matter they are now claiming was not anticipated by the Hostettler-Bailey combination reference, we think that the Patent Office has made a strong showing of prima facie obviousness, because, to paraphrase the board, it would not have been necessary for one skilled in the art to have gone any further than another part of Bailey to find the specified proportions of the appealed claims. With the discerning eye of hindsight, it certainly seems that it would not have required much skill in the art to realize that other, very similar, siloxane-oxyalkylene block copolymers, which Bailey also taught how to make, could also be **604 used in Hostettler's process for making polyurethane foams.[FN4]

FN4. The Bailey reference itself is totally silent regarding any use of its copolymer materials in the manufacture of polyurethane foams.

Appellants rely on two arguments to rebut this prima facie obviousness. First, they assert that their two Rule 132 affidavits establish that readctions employing their relatively small class of surfactants 'consistently gave smoothfer, finer celled foams of better appearance and *1323 often of improved height * * * with a plurality of reactants, catalysts and blowing agents' as compared with reactions employing surfactants taken from Hostettler's disclosure, and that, for the two runs in which these properties were compared, their surfactants also produced foams with improved tensile strengths and elongation characteristics. Second, they note (1) that, although

A part of the Hostettler grandparent application concerning the siloxane-oxyalkylene copolymers (R-399-405) is almost an exact copy of a portion of the Bailey et al. disclosure (R-333-334) and both have the same assignee, * * * the Hostettler grandparent application left out those portions of Bailey et al. which relate to siloxane-oxyalkylene copolymers in which the (C(n)H(2n)O)(z) groups have both oxyethylene and oxypropylene units * * *

and (2) that 'the main portion of the subject matter filed by Hostettler subsequent to his grandparent application is directed precisely to the use of the specific surfactants defined in the claims here on appeal.'[FN5] These facts, they argue, are evidence that the subject matter of their claims was not obvious to Hostettler, whom they dub 'a person of at least ordinary skill in the art,' and therefore are some evidence that the subject matter of their claims was not 'obvious' in the legal sense to the mythical men of ordinary skill in the art at the time they made their invention.

FN5. It may be of interest, though it has not influenced our decision in this case, that Hostettler denominated both of the successor applications to the present reference continuations-

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in-part.

The relevant portions of the two lengthy Rule 132 affidavits have been marshaled very effectively in tables filling four pages of appellants' main brief. We do not deem it necessary to reproduce those tables here, but we will describe them briefly. Appellants have compared polyurethane foams produced by the oneshot technique employing the surfactant recited in their narrow claims (i.e., a siloxane-oxyalkylene copolymer having chains consisting of 17 oxyethylene units and 13 oxypropylene units) with polyurethane foams produced by the one-shot technique employing surfactants taken from Hostettler (i.e., siloxane-oxyalkylene copolymers having chains consisting solely of oxyethylene units or solely of oxypropylene units[FN6]). The runs are divided into seven groups, allowing for variations in specific diisocyanate and polyol reactants, blowing agent (i.e., water and fluorocarbon),**1324** catalysts (i.e., both an amine catalyst and dibutyl tin dilaurate, the catalyst used by Hostettler), and comparison surfactants (i.e., a pure oxypropylene chain surfactant, two different pure oxyethylene chain surfactants, and a physical mixture of a pure oxypropylene and a pure oxyethylene**605** surfactant). The resulting foams are compared as to foam appearance, cell appearance, and foam height in the results taken from one affidavit and as to skin appearance, cell appearance, foam appearance, tensile strength, and percent ultimate elongation in the results taken from the other. In every group, the results employing appellants' surfactants are characterized by words such as 'Good' (for foam appearance) and 'Fine texture- Open' (for cell appearance),[FN7] while the results employing the surfactants taken from Hostettler's disclosure are characterized by words such as 'Poor, Collapsed' (for foam appearance) and 'Large, Course' (for cell appearance), and, in the two groups comparing skin appearance, the foams made with appellants' surfactants are said to have been 'Smooth' and 'Very smooth,' respectively, while the foams made with Hostettler's surfactants are said to have been 'Rough, Potted' in both cases. In four of the five groups where foam height was measured, the foams made with appellants' surfactants were higher than the foams made with Hostettler's, and, in the two groups in which tensile strength and percentage ultimate elongation were measured, the foams made with appellants' surfactants were significantly stronger than the foams made with Hostettler's surfactants.

> FN6. The examiner noted that the surfactants representing appellants' invention and the surfactants representing Hostettler's teachings in one of the affidavits differed in that the former had an ethyl group attached to the left-hand silicon while the latter had methyl group in the same place. The board did not mention this point, but the solicitor has argued that the difference makes the runs in that affidavit 'not comparable.' We must admit that this difference does detract from the weight to be accorded appellants' affidavit. However we agree with appellants that homology may be used by an applicant as well as against him and that, absent some reason to suspect that the small difference in these structures would affect the properties of the compound, the difference does not detract significantly from the strength of appellants' comparisons.
>
> FN7. In one case, the foam made by appellants' process is also said to suffer from an 'Internal Split,' which appellants admit is undesirable, and the solicitor has made much of this. While we agree that this detracts from the showing of unexpectedly advantageous results which appellants have made, we do not agree that it does so sufficiently to change the result in this case.

The board held that appellants' Rule 132 affidavits were 'not demonstrative of unobviousness' for what we take to be three separate reasons. First, they, thought that appellants had compared the wrong things, primarily because in only two of the comparison groups were appellants' surfactants compared with Hostettler surfactants having the same molecular weight. While appellants certainly did not isolate every possible variable and demonstrate that substitution of their surfactants invariably led to a superior result, we think their showing was adequate for an ex parte case absent any reason to expect a different result with some other set of variables.

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Compare In re Miller, 441 F.2d 689, 58 CCPA (1971). Second, the board objected to the use of subjective terms like 'good' and 'coarse' in the comparison on the ground that they are 'susceptible of varying interpretations.' Again, although we agree that objective comparisons are generally to be preferred to subjective comparisons, we cannot *1325 agree that comparisons made in such terms can be completely ignored, nor that they are, for that reason alone, entitled to little weight where the Patent Office has not sugg4sted a practicable objective standard for measuring the same variable. Furthermore, in this case appellants submitted specimens of cellular polyurethane foams made as described in one of the affidavits, the Patent Office therefore had the opportunity to verify the appellants' assertions therein to the extent possible with the limited technical facilities available there, and it has not been suggested that the characterizations in appellants' affidavit do not seem to be accurate. Third, the board stated that what objective comparisons were made indicated that 'other formulations changes known in the art may have as great or greater an effect than a change in surfactant.'This last objection, we think, reflects a misapprehension of what appellants were trying to prove by their affidavits. It is true, as the board pointed out, that the differences in tensile strength and percentage ultimate elongation between each of appellants' foams and the Hostettler foam with which it was compared were no greater than the differences in tensile strength and percentage ultimate elongation between the two runs of appellants' foam inter se (wiich two runs differed only in the polyol used). However, we accept appellants' affidavits as establishing**606 that the resulting foams were always better when the surfactant recited in appellants' narrow claims was used than when one of Hostettler's surfactants was used, everything else being the same, even though some combinations of variables including their surfactant were not as good as other combinations of variables, not including their surfactant and also different by at least one other variable. This is enough, in our view, to be 'demonstrative of unobviousness' in at least some of appellants' claims.

Appellants concede that all their objective evidence of non-obviousness concerns the specific surfactants recited in their narrow claims (i.e., those having 17 oxyethylene units and 13 oxyproplyene units), but argue that the ranges recited in their broad claims (i.e., those having 17 to 19 oxyethylene units and 11 to 15 oxypropylene units)

* * * are reasonable and limited expansions based on the results of the showings and afford appellants protection which is appropriate under the circumstances even though the showings do not rigorously cover the ranges.

However, in view of the very strong showing of prima facie obviousness made by the Patent Office in this case, we would not feel justified, on the basis of their showing as to the surfactants, in permitting appellants to expand their claims beyond the specific surfactants which they have shown to give unexpected and advantageous results. Compare *1326In re Landgraf, 436 F.2d 1046, 1050, 58 CCPA 929, (1971), and In re Byce, 378 F.2d 942, 945, 54 CCPA 1454, 1458-1459 (1967).

Appellants also argue that all claims which recite the amine catalayst should be held patentable because

The fact that the secondary references describe the use of amines in various other polyurethane foam systems is of no particular significance since none of the secondary references provides a basis for concluding that appellants' choice of surfactant and amine catalyst would produce unexpected results.

However, the 'unexpected results' they are referring to are simply that Hostettler's method works with amine as well as tin catalysts; they do not argue that their data show that their amine catalysts produce better results than Hostettler's tin catalysts. They do argue that 'the Hostettler disclosure * * * (leads) in the opposite direction (from amine catalysts),' but they show only that Hostettler taught that tin catalysts would work, not that he taught that amine catalysts would not. Under the circumstances, we agree with the solicitor that appellants have not rebutted the showing of prima facie obviousness the Patent Office made with respect to the case of appellants' amine catalysts.

There remains one final argument, which the solicitor

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presses with great vigor and for which he finds some support in the board's opinion, as to why appellants are not entitled to their claims even if the claims are found to be non-obvious in view of the prior art. This argument is that appellants' application states only that the particular surfactants recited in the appealed claims are 'preferred' and does not indicate that the choice of these particular surfactants, as opposed to others appellants disclosed and formerly claimed, is 'critically significant.' He summarizes this argument as follows:

In view of the prior adjudications by this Court, it is well established law that claimed features, proportions or values which are not disclosed as critical or critically significant and/or are described in the application only as being merely referred 'cannot be considered as critical', i. e., patentability cannot be predicated thereon.In re Gardiner, 171 F.2d 313, 36 C.C.P.A. 748, 751:In re Honnig, 193 F.2d 191, 39 C.C.P.A. 740, 743;in re Bourdon, 240 F.2d 358, 44 C.C.P.A. 740, 743;In re Shepard, 319 F.2d 194, 50 C.C.P.A. 1439, 1445.

**607 Appellants concede that, as the result of the vicissitudes of prosecution, they are now claiming much less than they originally did and that, in fact, what they are now claiming was originally only the preferred class within much broader claims, but they argue that the essential question in cases of this sort is whether the inventor has, in fact, rebutted any prima facie obviousness with objective evidence of non-obviousness and not whether his original application happens to use the 'correct' modifying adjectives (i.e., 'critical' rather than 'preferred') to describe the subject matter eventually claimed.

*1327 Actually, this court in In re Bourdon and In re Shepard said only:

As was held in In re Gardiner, 171 F.2d 313, 36 C.C.P.A. (patents) 748, values which are described in an application only as being preferred cannot ordinarily be held to be critical.319 F.2d at 199, 50 C.C.P.A. at 1445;240 F.2d at 360, 44 C.C.P.A. at 743.

Furthermore, we agree with appellants' argument that

It is quite clear from the Shepherd and other decisions cited by the Commissioner that the Court did not refuse to consider or give appropriate weight to affidavits because the essential novelty of the claimed subject matter was disclosed in the application only as 'preferred.' Rather after a complete study of the facts the Court found that the differences between the prior art and the claimed invention established by the showings made were not sufficient to establish unobviousness and patentability. In other words, the showing were found to be inadequate.

[5][6][7] However, regardless of what this court may have said in the past, it is now our view that, in those cases where the applicants have attempted to rebut a showing of the prima facie obviousness of the subject matter claimed by the introduction of objective evidence of non-obviousness, both we and the tribunals of the Patent Office must give full consideration to the applicant's evidence and reach a decision on the question of non-obviousness on the basis of the relative strength of the applicant's showing and the prima facie case made by the Patent Office.In re Orfeo, 440 F.2d 439, 441, 58 CCPA (1971); Graham v. John Deere Co., 383 U.S. 1, 17-18, 86 S.Ct. 684, 15 L.Ed.2d 545 (1966). Appellants' evidence may not be disregarded simply because of the manner in which the now claimed processes were denominated in the original application. To rule otherwise would let form triumph over substance, substantially eliminating the right of an applicant to retreat to an otherwise patentable species merely because he erroneously thought he was first with the genus when he filed. Cf. In re Ruff, 256 F.2d 590, 597, 45 CCPA 1037, 1049 (1958). Since the patent law provides for the amendment during prosecution of claims, as well as the specification supporting claims, U.S.C. 132, it is clear that the reference to 'particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention' in the second paragraph of 35 U.S.C. 112 does not prohibit the applicant from changing what he 'regards as his invention' (i.e., the subject matter on which he seeks patent protection) during the pendency of his application. Cf. In re Brower, 433 F.2d 813, 817, 58 CCPA 724 (1970) (fact that claims in continuation application were directed to subject matter

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which appellants had not regarded as part of their invention when the parent application was filed held not to prevent the continuation application from receiving benefit of parent's date).

*1328 DECISION

For the foregoing reasons, the rejection of claims 11 and 26 is reversed and the rejection of claims 6, 8-10, 19-21, and 23 is affirmed.

Modified.

**608 BALDWIN, Judge (concurring).

While I adhere to the bulk of the court's opinion, I wish to express my disagreement with that section which finds error in the rejection of claims 6, 20 and 23 (not reciting any catalyst) under 35 U.S.C. 102 as anticipated by the Hostettler reference. I would affirm this rejection because I believe the whole of the Bailey disclosure relating to the preparation of siloxane-oxyalkylene block copolymers is incorporated by reference into Hostettler.

The logic of the majority approach fails, I believe, to take into consideration adequately the fact that, on this record, there is no reason to believe that one of ordinary skill, reading Hostettler's disclosure, would know how to make any of the critical block copolymers. Accordingly, whether or not that mythical man would presume from Hostettler's primary disclosure that mixtures of oxyalkylenes in the copolymers were not contemplated, upon going to Bailey as instructed he would find out conclusively that such mixtures were also included. As the board here indicated, therefore, the effective disclosure of the Hostettler reference clearly includes utilization of the copolymer described in Example 1(a) of Bailey as the surfactant in the Hostettler process.

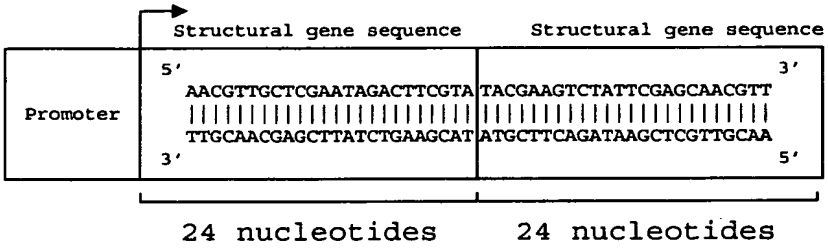
Cust. & Pat.App. 1971.
Application of Saunders
58 C.C.P.A. 1316, 444 F.2d 599, 170 U.S.P.Q. 213

END OF DOCUMENT

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# EXHIBIT F

A double-stranded genetic construct




Transcription

5′ 3′

AACGUUGCUCGAAUAGACUUCG**UAUA**CGAAGUCUAUUCGAGCAACGUU

("Single self-complementary RNA strand")

Self-hybridization

